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(54) Title: METHODS FOR MODULATING T CELL RESPONSES BY MANIPULATING INTRACELLULAR SIGNAL TRANSDUCTION

(57) Abstract

Methods for modulating T cell responses by manipulating intracellular signals associated with T cell costimulation are disclosed. The methods involve inhibiting or stimulating the production of at least one D3-phosphoinositide in a T cell. Production of D3-phosphoinositides can be manipulated by contacting a T cell with an inhibitor or activator of phosphatidylinositol 3-kinase. Inhibitors of phosphatidylinositol 3-kinase for use in the methods of the invention include wortmannin and quercetin, or derivatives or analogues thereof. The methods of the invention can further comprise modulating other intracellular signals associated with costimulation, such as protein tyrosine phosphorylation, for example by modulating the activity of a protein tyrosine kinase or a protein tyrosine phosphatase in the T cell. Inhibition of a T cell response in accordance with the disclosed methods is useful therapeutically in situations where it is desirable to inhibit an immune response to an antigen(s), for example in organ or bone marrow transplantation and autoimmune diseases. Alternatively, stimulation of a T cell response in accordance with the disclosed methods is useful therapeutically to enhance an immune response to an antigen(s), for example to stimulate an anti-tumor response in a subject with a tumor, to stimulate a response against a pathogenic agent or increase the efficacy of vaccination. Novel screening assays for identifying inhibitors or activators of phosphatidylinositol 3-kinase, which can be used to inhibit or stimulate a T cell response, are also disclosed.

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METHODS FOR MODULATING T CELL RESPONSES BY MANIPULATING INTRACELLULAR SIGNAL TRANSDUCTION

Background of the Invention

The induction of antigen-specific T cell responses involves multiple interactions between cell surface receptors on T cells and ligands on antigen presenting cells (APCs). The primary interaction is between the T cell receptor (TCR)/CD3 complex on a T cell and a major histocompatibility complex (MHC) molecule/antigenic peptide complex on an antigen presenting cell. This interaction triggers a primary, antigen-specific, activation signal in the T cell. In addition to the primary activation signal, induction of T cell responses requires a second, costimulatory signal. In the absence of proper costimulation, TCR signalling can induce a state of anergy in the T cell. Subsequent appropriate presentation of antigen to an anergic T cell fails to elicit a proper response (see Schwartz, R.H. (1990) *Science* 248:1349).

A costimulatory signal can be triggered in a T cell through a T cell surface receptor, such as CD28. For example, it has been demonstrated that suboptimal polyclonal stimulation of T cells (e.g. by anti-CD3 antibodies or phorbol ester, either of which can provide a primary activation signal) can be potentiated by crosslinking of CD28 with anti-CD28 antibodies (Linsley, P.S. et al. (1991) *J. Exp. Med.* 173:721; Gimmi, C.D. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:6575). Moreover, stimulation of CD28 can prevent the induction of anergy in T cell clones (Harding, F. A. (1992) *Nature* 356:607-609). Natural ligands for CD28 have been identified on APCs. CD28 ligands include members of the B7 family of proteins, such as B7-1(CD80) and B7-2 (B70) (Freedman, A.S. et al. (1987) *J. Immunol.* 137:3260-3267; Freeman, G.J. et al. (1989) *J. Immunol.* 143:2714-2722; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174:625-631; Freeman, G.J. et al. (1993) *Science* 262:909-911; Azuma, M. et al. (1993) *Nature* 366:76-79; Freeman, G.J. et al. (1993) *J. Exp. Med.* 178:2185-2192). In addition to CD28, proteins of the B7 family have been shown to bind another surface receptor on T cells related to CD28, termed CTLA4, which may also play a role in T cell costimulation (Linsley, P.S. (1991) *J. Exp. Med.* 174:561-569; Freeman, G.J. et al. (1993) *Science* 262:909-911).

The elucidation of the receptor:ligand relationship of CD28/CTLA4 and the B7 family of proteins, and the role of this interaction in costimulation, has led to therapeutic approaches involving manipulation of the extracellular interactions of surface receptors on T cells which bind costimulatory molecules. For example, a CTLA4Ig fusion protein, which binds to both B7-1 and B7-2 and blocks their interaction with CD28/CTLA4, has been used to inhibit rejection of allogeneic and xenogeneic grafts (see e.g., Turka, L.A. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:11102-11105; Lenschow, D.J. et al. (1992) *Science* 257:789-792). Similarly, antibodies reactive with B7-1 and/or B7-2 have been used to inhibit T cell proliferation and IL-2 production *in vitro* and inhibit primary immune responses to antigen *in vivo* (Hathcock K.S. et al. (1993) *Science* 262:905-907; Azuma, M. et al. (1993) *Nature*

366:76-79; Powers, G.D. et al. (1994) *Cell. Immunol.* 153:298-311; Chen C. et al. (1994) *J. Immunol.* 152:2105-2114). Together, these studies indicate that T cell surface receptors which bind costimulatory molecules such as B7-1 and B7-2 are desirable targets for manipulating immune responses.

While the extracellular interactions between CD28/CTLA4 with their ligands have been characterized in some detail, little is known regarding the intracellular events that occur in a T cell following ligation of these molecules. T cell costimulation is thought to involve an intracellular signal transduction pathway distinct from signalling through the TCR since the costimulatory pathway is resistant to the inhibitory effects of cyclosporin A (see June, C.H. et al. (1990) *Immunology Today* 11:211-216) Protein tyrosine phosphorylation has been shown to occur in T cells upon CD28 ligation and it has been demonstrated that a protein tyrosine kinase inhibitor, herbimycin A, can inhibit CD28-induced IL-2 production (Vandenberghe, P. et al. (1992) *J. Exp. Med.* 175:951-960; Lu, Y. et al. (1992) *J. Immunol.* 149:24-29).

Studies indicate that the CD28 receptor can trigger signals in common with the antigen receptor as well as signals that are independent of the antigen receptor. Further the degree of CD28 receptor oligomerization is an important determinant in CD28-mediated signal transduction (Linsley, P.S. and Ledbetter, J.A. (1993) *Annu. Rev. Immunol.* 11:191 and Ledbetter, J.A. et al. (1990) *Blood* 75:1531). Studies using anti-CD28 and anti-CD3 monoclonal antibodies demonstrate that calcium signaling, phospholipase C (PLC) activation, increased tyrosine kinase activity, and $p21^{ras}$ activation all occur early after receptor engagement (reviewed in June, C.H. et al. (1994) *Immunol. Today* 15: 321 and Rudd, C.E. et al. (1994) *Immunol. Today* 15:225). The protein tyrosine kinases $p72^{itk}$ and $p56^{lck}$ (August, A. and Dupont, B. (1994) *Biochem. Biophys. Res. Commun.* 199:1466 and August, A. et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:9347) have been reported to be activated or physically associated with both CD28 and the T cell antigen receptor. The CD28-mediated calcium, $p21^{ras}$ and $p70^{s6}$ kinase signals are more prominent in T cell blasts than on resting T cells, and are temporally delayed in comparison to antigen receptor signals (Ledbetter, J.A. et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:1384; Nunes, J.S. et al. (1993) *Biochem. J.* 293:835; Siegel, J.N. et al. (1993) *J. Immunol.* 151:4116; and Pai, S.V. et al. (1994) *J. Immunol.* 24:2364). Furthermore, while both $CD8^+$ T cells and $CD4^+$ T cells respond to CD28 signals, anti-CD28-induced calcium signals are primarily restricted to $CD4^+$ T cells (Abe, R.P. et al. (1995) *J. Immunol.* in press). Together these studies indicate that CD28 signal transduction is coupled to several signal transduction cascades and that the nature of the signal is regulated during T cell development.

Summary of the Invention

This invention relates to the regulation of T cell responses by manipulation of intracellular signal transduction. In particular, intracellular signalling events which occur

upon costimulation of a T cell are manipulated. The invention encompasses methods for modulating T cell responses by inhibiting or stimulating one or more intracellular signals which result from ligation of a surface receptor on a T cell which binds a costimulatory molecule. It has now been discovered that CD28 receptor stimulation leads to the production of D3-phosphoinositides within a T cell. Moreover, it has been discovered that inhibition of the activity of phosphatidylinositol 3-kinase in a T cell can inhibit T cell responses, such as lymphokine production and cellular proliferation. These discoveries indicate a functional role for D3-phosphoinositides in a costimulatory signal transduction pathway and provide phosphatidylinositol 3-kinase as an intracellular target for modulation of T cell responses. Accordingly, intracellular signalling events involving D3-phosphoinositides can be modulated either to inhibit a costimulatory signal and thereby induce T cell unresponsiveness, or to trigger a costimulatory signal and thereby generate a T cell response. In addition, novel screening assays for identifying inhibitors or activators of phosphatidylinositol 3-kinase, which can be used to inhibit or stimulate a T cell response, are within the scope of the invention.

One aspect of the invention pertains to methods for inhibiting a response by a T cell which expresses a surface receptor that binds a costimulatory molecule. These methods involve contacting the T cell with an agent which inhibits production of D3-phosphoinositides in the T cell. In one embodiment, the agent is an inhibitor of a phosphatidylinositol 3-kinase, such as the fungal metabolite wortmannin or the bioflavenoid quercetin, or derivatives or analogues thereof (e.g. LY294002). In another embodiment of the method of the invention, the T cell is contacted with at least one additional agent which inhibits a different intracellular signal associated with costimulation, such as protein tyrosine phosphorylation. For example, the T cell can be contacted both with an inhibitor of phosphatidylinositol 3-kinase and with an inhibitor of a protein tyrosine kinase. A preferred inhibitor of a protein tyrosine kinase is herbimycin A. Alternatively, protein tyrosine phosphorylation can be inhibited in a T cell by a tyrosine phosphatase or an activator of a tyrosine phosphatase. In this embodiment, the T cell can be contacted with an inhibitor of phosphatidylinositol 3-kinase and with a molecule, e.g., an antibody, which binds to and activates a cellular tyrosine phosphatase, such as CD45 or Hcph. In another preferred embodiment of the method of the invention, the T cell is contacted with an inhibitor of a protein serine kinase or an activator of a serine phosphatase.

The invention also provides methods for inducing unresponsiveness to an antigen in a T cell by triggering a primary, antigen-specific signal in a T cell while interfering with an intracellular signal associated with costimulation in the T cell. As a result of interfering with costimulatory signal transduction, the T cell fails to receive a proper costimulatory signal in the presence of the antigen and antigen-specific unresponsiveness is induced in the T cell. To induce T cell unresponsiveness, an antigen-specific T cell is contacted with the antigen in a form suitable for stimulation of a primary activation signal in the T cell, together with an

agent which inhibits production of D-3 phosphoinositides in the T cell. For example, a T cell can be contacted with an antigen presented by an APC together with an inhibitor of phosphatidylinositol 3-kinase, such as wortmannin or quercetin or derivatives or analogues thereof (e.g. LY294002). Additionally, other intracellular signals associated with costimulation, such as protein tyrosine phosphorylation and/or protein serine phosphorylation, can be inhibited in the T cell.

Methods for inhibiting T cell responses and for inducing T cell unresponsiveness are useful in situations where it is desirable to down-modulate an immune response, for example in a transplant recipient (e.g., of an organ graft or bone marrow graft etc.) or a subject suffering from an autoimmune disease or other disorder associated with an abnormal immune response. An agent which inhibits signal transduction associated with costimulation (e.g., an inhibitor of inositol phosphate 3-kinase) can be administered to a subject or, alternatively, T cells can be obtained from the subject, treated *in vitro* as described herein and administered to the subject.

Another aspect of the invention pertains to methods for stimulating a response by a T cell which has received a primary activation signal and expresses a surface receptor that binds a costimulatory molecule. These methods involve contacting the T cell with an agent which stimulates production of D-3 phosphoinositides in the T cell, such as an activator of phosphatidylinositol 3-kinase. In another embodiment, the T cell is contacted with an agent which stimulates production of D-3 phosphoinositides and at least one additional agent which stimulates a different intracellular signal associated with costimulation, such as protein tyrosine phosphorylation. For example, the T cell can be contacted with an activator of phosphatidylinositol 3-kinase together with an activator of a protein tyrosine kinase, such as pervanadate. In another embodiment of the invention, the T cell is further contacted with a an agent which activates a protein serine kinase. Alternatively, an inhibitor of a cellular phosphatase, such as CD45 or Hcph, can be used in conjunction with a PI3K activator. In yet another embodiment of the invention, an antigen-specific T cell response is stimulated by contacting an antigen-specific T cell with the antigen together with an agent which stimulates production of D-3 phosphoinositides in the T cell, thereby stimulating both a primary activation signal and a costimulatory signal in the T cell. In another embodiment of the invention, the antigen-specific T cell response is further stimulated by contacting the antigen-specific T cell with one or more agents which activate protein tyrosine and/or protein serine kinases.

Methods for stimulating T cell responses are useful in situations where it is desirable to up-regulate an immune response. For example a response against a tumor in a tumor-bearing subject can be stimulated or a response against a pathogen (e.g., a bacteria, a virus, such as HIV, fungus, parasite etc.) in a subject can be stimulated. Additionally, the methods can be used to enhance the efficacy of vaccination. An agent which stimulates an intracellular signal associated with costimulation (e.g., an activator of inositol phosphate 3-

kinase) can be administered to a subject or, alternatively, T cells can be stimulated *in vitro* and then administered to a subject.

Another aspect of the invention pertains to screening assays for identifying inhibitors or activators of a phosphatidylinositol 3-kinase. In one embodiment, a T cell which expresses a cell surface receptor (e.g., CD28) which binds a costimulatory molecule is utilized. To identify an inhibitor, an intracellular signal transduction pathway associated with the receptor in the T cell is stimulated in the presence of an agent to be tested and an inhibitor is identified based upon its ability inhibit production of at least one D-3 phosphoinositide in a T cell. To identify an activator, the T cell is contacted with an agent to be tested and an activator is identified based upon its ability to stimulate production of at least one D-3 phosphoinositide in a T cell.

The invention further pertains to a novel tyrosine-phosphorylated protein of approximately 67 kDa which is associated with CD28 in T cells stimulated through CD28. Methods for blocking activation of the protein, or its interaction with CD28, or alternatively, methods for activating the 67K protein or stimulating its interaction with CD28 are also contemplated herein.

Brief Description of the Drawings

Figure 1 is a graphic representation of the production of phosphatidylinositol(3,4,5)-triphosphate in $CD28^+$ T cells (Jurkat cells) following stimulation of the cells with medium, an anti-CD3 antibody or an anti-CD28 antibody, demonstrating distinct kinetics of phosphatidylinositol 3-kinase activation upon stimulation through CD3 or CD28.

Figure 2 is a grahic representation of the production of phosphatidylinositol(3,4,5)-triphosphate in $CD28^+$ T cells (Jurkat cells) following stimulation of the cells with CHO cells transfected to express B7-1 or B7-2, demonstrating distinct kinetics of phosphatidylinositol 3-kinase activation upon stimulation with B7-1 or B7-2.

Figure 3 is a graphic representation of the effect of various concentrations of wortmannin (0-100 mM) on production of phosphatidylinositol(3,4,5)-triphosphate in $CD28^+$ T cells (Jurkat cells) following stimulation of the cells with CHO cells transfected to express B7-2.

Figure 4 is a graphic representation of the effect of wortmannin or herbimycin on calcium influx in T cells induced by ligation of CD28 with an anti-CD28 antibody.

Figure 5 is a series of flow cytometric profiles from a cell-conjugate assay in which Jurkat cells were incubated either CHO-neo, CHO-B7-1 or CHO-B7-2 cells. Calcium flux is indicated on the Y-axis and cell conjugation is indicated on the X-axis.

Figure 6 is a graphic representation of the effect of membrane-bound B7-1 and B7-2, in combination with an anti-CD3 antibody, on IL-2 production by purified human peripheral blood T cells, demonstrating a dose dependent increase in IL-2 production by costimulation with either B7-1 or B7-2.

Figure 7A is a graphic representation of the effect of wortmannin treatment (1 nM to 1 mM) on IL-2 production by resting human T cells 24 hours after stimulation of the cells with media, immobilized anti-CD3 + CHO-B7-1, immobilized anti-CD3 + CHO-B7-2, PMA + CHO-B7-1 or PMA + CHO-B7-2.

Figure 7B is a graphic representation of the percent inhibition by wortmannin (1 to 100 nM) of IL-production by human T cells stimulated for 24 hours with anti-CD3 antibody (OKT3) together with CHO cells expressing B7-1, B7-2 or both B7-1 and B7-2, or T cells stimulated with PMA together with CHO cells expressing B7-1 or B7-2.

Figure 8 is a graphic representation of the effect of herbimycin pretreatment on the production of phosphatidylinositol (3,4,5)-triphosphate by Jurkat cells stimulated with anti-CD28 mAb (9.3), CHO, CHO-B7-1, or CHO-B7-2 cells.

Figure 9 represents a thin layer chromatography showing the amount of phosphatidylinositol(3,4,5)-triphosphate produced by Jurkat cells at different time points after stimulation with anti-CD28 mAb (9.3) or CHO-B7-1 (B7) cells in the presence (+) or absence (-) of herbimycin.

Figure 10A is a graphic representation of IL-2 secretion by peripheral blood T cells stimulated with PMA alone or with CHO-B7-1 (PMA + CHOB71), CHO-B7-2 (PMA + CHOB7-2), 9.3 mAb in solution (PMA + 9.3), 9.3 mAb on beads (PMA + 9.3 beads), ionomycin (PMA + Iono), or ionomycin and soluble 9.3 mAb (PMA + Iono +9.3) in the presence or absence of herbimycin A.

Figure 10B is a graphic representation of IL-2 secretion by peripheral blood T cells stimulated with anti-CD3 (OKT3) alone or with CHO-B7-1 (OKT3 + CHOB71), CHO-B7-2 (OKT3 + CHOB72), or mAb 9.3 (OKT3 + 9.3) in the presence (+Iono) or absence of ionomycin.

Figure 11 represents an autoradiography of an immunoblot showing that a phosphotyrosine protein of approximately 67 kDa is coimmunoprecipitated with an anti-CD28 antibody.

Detailed Description of the Invention

This invention features methods for regulating T cell responses by modulating intracellular signals generated in a T cell upon costimulation, e.g. by binding of a surface receptor on the T cell to a costimulatory molecule. In particular, the invention pertains to modulation of the production of D-3 phosphoinositides in a T cell to inhibit or stimulate a costimulatory signal to thereby inhibit or stimulate T cell responses. Inhibition of a costimulatory signal by interfering with signal transduction associated with costimulation can further be used to induce T cell unresponsiveness. The invention is based, at least in part, on the discovery that stimulation of a T cell through the CD28 surface receptor leads to the production of D-3 phosphoinositides in a T cell and that an inhibitor of a phosphatidylinositol 3-kinase (also referred to herein as PI3K) inhibits production of D-3 phosphoinositides in the

T cell upon CD28 ligation. The invention is further based, at least in part, on the discovery that inhibition of PI3K activity in a T cell inhibits T cell responses, such as cytokine production and cellular proliferation.

Accordingly, one aspect of the invention pertains to methods for inhibiting a response by a T cell by interfering with intracellular signal transduction associated with signal transduction. In one embodiment, an intracellular signal is inhibited by contacting a T cell expressing a cell surface receptor that binds a costimulatory molecule with an agent which inhibits production of D-3 phosphoinositides in the T cell. The term "a T cell expressing a cell surface receptor that binds a costimulatory molecule" is intended to encompass T cells expressing CD28 and/or CTLA4, or other receptor capable of binding a costimulatory molecule such as B7-1, B7-2 or other B7 family member.

A "response" by a T cell is intended to encompass T cell responses that occur upon triggering of a primary activation signal and a costimulatory signal in the T cell, and includes lymphokine production (e.g., IL-2 production) and T cell proliferation. Inhibition of a T cell response may involve complete blocking of the response (i.e., a lack of a response) or a reduction in the magnitude of the response (i.e., partial inhibition of the response).

The term "D-3 phosphoinositides" is intended to include derivatives of phosphatidylinositol that are phosphorylated at the D-3 position of the inositol ring and encompasses the compounds phosphatidylinositol(3)-monophosphate (PtdIns(3)P), phosphatidylinositol(3,4)-bisphosphate (PtdIns(3,4)P_2), and phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)P_3).

D-3 phosphoinositides are generated intracellularly by the activity of a phosphatidyl-inositol 3-kinase (PI3K). PI3K is a heterodimer composed of an 85 kDa subunit that binds tyrosyl-phosphorylated proteins via its SH2 domains and a 110 kDa catalytic subunit. PI3K was first identified as a lipid kinase that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol, PtdIns (4)P, and PtdIns(4,5)P2. Two recent studies have demonstrated that PI3K is in fact a dual-specificity kinase that possesses both lipid and serine kinase activities (Dhand, R. et al. (1994) *EMBO J.* 13:522 and Carpenter, C.L. et al. (1993) *Mol. Cell Biol.* 13:1657).

Accordingly, in one embodiment, the agent which inhibits production of a D-3 phosphoinositide in the T cell is an agent which inhibits the activity of a PI3K. A preferred agent which inhibits PI3K activity in a T cell is the fungal metabolite wortmannin, or derivatives or analogues thereof. Wortmannin is a potent PI3K inhibitor derived from *T. wortmannii* (Kyowa Hakko Kohyo Co. Ltd.) or from *P. fumiculosum* (Sigma). Wortmannin derivatives or analogues include compounds structurally related to wortmannin which retain the ability to inhibit PI3K and T cell responses. Examples of wortmannin derivatives and analogues are disclosed in Wiesinger, D. et al. (1974) *Experientia* 30:135-136; Closse, A. et al. (1981) *J. Med. Chem.* 24:1465-1471; and Baggiolini, M. et al. (1987) *Exp. Cell Res.* 169:408-418. Another inhibitor of PI3K activity that can be used is the bioflavenoid

quercetin, or derivatives or analogues thereof. Quercetin derivatives or analogues include compounds structurally related to quercetin that retain the ability to inhibit PI3K and inhibit T cell responses. Examples of quercetin derivatives and analogues are disclosed in Vlahos, C.J. et al. (1994) *J. Biol. Chem.* 269:5241-5284. A preferred quercetin derivative which inhibits PI3K activity is LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, Lilly Indianapolis, IN) (described in Vlahos et al. cited *supra*). Alternatively, other inhibitors of PI3K, for example those identified by methods described below, can be used.

Another aspect of the invention involves inhibiting a response by a T cell by interfering with two or more intracellular signalling events associated with costimulation. For example, CD28 stimulation has been shown to result in protein tyrosine phosphorylation in the T cell (see e.g., Vandenberghe, P. et al. (1992) *J. Exp. Med.* 175:951-960; Lu, Y. et al. (1992) *J. Immunol.* 149:24-29). Accordingly, in one embodiment, a T cell response is inhibited by contacting a T cell with a first agent which inhibits production of at least one D-3 phosphoinositide in the T cell and with a second agent which inhibits tyrosine phosphorylation in the T cell. For example, the T cell can be contacted both with an agent which inhibits PI3K activity and with an agent which inhibits protein tyrosine kinase activity. A preferred protein tyrosine kinase inhibitor is one which inhibits *src* protein tyrosine kinases. In one embodiment, the *src* protein tyrosine kinase inhibitor is herbimycin A, or a derivative or analogue thereof. Derivatives and analogues of herbimycin A include compounds which are structurally related to herbimycin A and retain the ability to inhibit the activity of protein tyrosine kinases. In another embodiment, the agent which inhibits protein tyrosine phosphorylation is a protein tyrosine phosphatase or an activator of a protein tyrosine phosphatase. By increasing the tyrosine phosphatase activity in a T cell, the net amount of protein tyrosine phosphorylation is decreased. The protein tyrosine phosphatase can be a cellular protein tyrosine phosphatase within the T cell, such CD45 or Hcph. The activity of a cell surface tyrosine phosphatase on a T cell can be activated by contacting the T cell with a molecule which binds to the phsophatase and stimulates its activity. For example, an antibody directed against CD45 can be used to stimulate tyrosine phosphatase activity in a T cell expressing CD45 on its surface. Accordingly, in one embodiment, the agent which inhibits protein tyrosine phosphorylation within the T cell is an anti-CD45 antibody, or a fragment thereof which retains the ability to stimulate the activity of CD45. Examples of antibody fragments include Fab and F(ab')2 fragments. Antibodies, or fragments thereof, can be provided in a stimulatory form, for example multimerized or immobilized etc.

Other intracellular signals associated with costimulation can be inhibited together with inhibition of D-3 phosphoinositide production to inhibit T cell responses. For example, CD28 ligation has been associated with increased phospholipase C activity (see e.g., Nunes, J. et al. (1993) *Biochem. J.* 293:835-842) and increased intracellular calcium levels (see e.g. Ledbetter, J.A. et al. (1990) *Blood* 75:1531-1539 and the Examples). Accordingly, T cells can be contacted with both a first agent which inhibits PI3K activity and a second agent

which inhibits phospholipase C activity and/or inhibits increases in intracellular calcium levels. As demonstrated in the Examples, the tyrosine kinase inhibitor herbimycin A also inhibits CD28-induced calcium flux in T cells.

Furthermore, as demonstrated in the Examples, stimulation through CD28 can result in a herbimycin resistant production of IL-2 by the T cells, suggesting that the signal transduction pathway implicated involves additional protein kinases, such as protein serine kinases. Studies have shown that protein serine kinases are involved in signal transduction pathways associated with CD28 (Siegel, J.N. et al. (1993) *J. Immunol.* 151:4116-4127 and Pai, S.V. et al. (1994) *J. Immunol.* 24:2364). Thus, in a specific embodiment of the invention, T cell responses are inhibited by contacting T cells with an agent which inhibits PI3K activity and an agent which inhibits serine kinases. In another embodiment, such T cells are further contacted with an agent which inhibits protein tyrosine kinases. In yet other embodiments of the invention a T cell response is inhibited by contacting the T cells with various combinations of agents which inhibit PI3K, protein tyrosine kinases, protein serine kinases, phospholipase C activity, or an agent which inhibits increases in intracellular calcium.

T cell responses can be inhibited according to the methods of the invention either *in vitro* or *in vivo*. For example, an agent which inhibits D-3 phosphoinositide production in a T cell can be administered to a subject at a dose and for a period of time sufficient to inhibit T cell responses. Alternatively, T cells can be obtained from a subject, contacted with the agent *in vitro* and readministered to the subject. The term subject is intended to include animals in which immune responses occur, e.g., mammals, including humans, monkeys, dogs, cats, rabbits, rats, mice, and transgenic species thereof. Subjects in which T cell responses can be inhibited include subjects in which it is desirable to downmodulate an immune response. Downmodulation is intended to encompass both partial and complete inhibition of T cell responses, such as lymphokine production and T cell proliferation. The methods are applicable, for example, to a subject suffering from an autoimmune disease or other disorder associated with an abnormal immune response, or a transplant recipient, such as a recipient of a bone marrow transplant or other organ transplant.

In one embodiment of the invention, a costimulatory signal is inhibited in a T cell to induce antigen-specific T cell unresponsiveness. Accordingly, another aspect of the invention pertains to methods for inducing T cell unresponsiveness to an antigen. The term "T cell unresponsiveness" as used herein refers to a reduction in or lack of a T cell response (e.g., proliferation, lymphokine secretion or induction of effector functions) by a T cell upon exposure to an antigen (or antigenic portion) to which the T cell has been rendered unresponsive. The terms "T cell unresponsiveness" and "T cell anergy" are used interchangeably herein. T cell unresponsiveness to an antigen can be induced by triggering an antigen-specific primary activation signal in the T cell (e.g., activation through the TCR/CD3 complex) in the absence of a costimulatory signal. In the method of the invention,

a costimulatory signal is blocked in a T cell by contacting the T cell with an agent which interferes with an intracellular signal associated with costimulation. Specifically, T cell unresponsiveness to an antigen can be induced by contacting an antigen-specific T cell (i.e., a T cell expressing a TCR which recognizes the antigen) with the antigen in a form suitable to trigger a primary activation signal in the T cell in the presence of an agent which inhibits production of D-3 phosphoinositides in the T cell to inhibit a costimulatory signal. The antigen can be, for example, an autoantigen which stimulates an autoimmune reaction or an alloantigen which stimulates rejection of transplanted cells. Preferably, the agent which inhibits production of D-3 phosphoinositides inhibits the activity of PI3K in the T cell, such as wortmannin or quercetin, or a derivative or analogue thereof (e.g., LY294002). Additional agents which inhibit other intracellular signals associated with costimulation (as discussed above) can also be used in conjunction with an agent which inhibits production of D-3 phosphoinositides in the T cell. For example, the T cell can be contacted with a PI3K inhibitor together with a protein tyrosine kinase inhibitor, such as herbimycin A.

To induce T cell unresponsiveness, an antigen-specific T cell is contacted with an antigen in a form suitable to trigger a primary activation signal in the T cell, which means that the antigen is presented to the T cell such that a signal is triggered in the T cell through the TCR/CD3 complex. For example, the antigen can be presented to the T cell by an antigen presenting cell in conjunction with an MHC molecule. A syngeneic antigen presenting cell, such as a B cell, macrophage, monocyte, dendritic cell, Langerhans cell, or other cell which can present antigen to a T cell, can be incubated with the T cell in the presence of the antigen such that the antigen presenting cell presents the antigen to the T cell. Alternatively, to induce anergy to alloantigens, the T cell can be incubated with an allogeneic cell, which presents alloantigens to the T cell.

To induce T cell unresponsiveness to an antigen *in vivo*, an agent which inhibits production of D-3 phosphoinositides in a T cell is administered to a subject at a dose and for a period of time sufficient to induce T cell unresponsiveness to the antigen. Following administration of the agent, antigen-specific T cells are contacted with the antigen endogenously (for example, an autoantigen expressed by cells endogenously). Alternatively, T cell unresponsiveness to an antigen can be induced *in vitro*. In this case, T cells are obtained from a subject, contacted *in vitro* with the antigen together with the agent to induce antigenic unresponsiveness, and then readministered to the subject. For example, T cells obtained from a transplant recipient can be contacted with allogeneic cells from a graft donor together with an agent which inhibits D-3 phosphoinositide production in the T cells (e.g., wortmannin, quercetin, LY294002) and/or with one or more agents which inhibit one or more other intracellular signals associated with costimulation prior to transplantation of the graft into the recipient to induce alloantigen-specific T cell unresponsiveness. The recipient T cells which have been rendered unresponsive to the donor antigens are then readministered to the recipient. Alternatively, in the case of bone marrow transplantation, bone marrow to be

transplanted (including any residual T cells) can be contacted *in vitro* with allogeneic cells from the bone marrow recipient together with an agent which inhibits D-3 phosphoinositide production and/or other intracellular signals associated with costimulation to induce unresponsiveness in the donor T cells to recipient alloantigens. This pretreatment can be performed to inhibit graft versus host disease.

The methods for inducing T cell unresponsiveness can be applied therapeutically in situations where it is desirable to downmodulate an immune response, such as transplantation, including organ transplants and bone marrow transplants (as discussed above), and autoimmune diseases and other disorders associated with an abnormal immune response. Examples of autoimmune diseases or disorders associated with an inappropriate or abnormal immune response include rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, allergies, contact dermatitis, psoriasis, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, multiple sclerosis, allergic encephalomyelitis, systemic lupus erythematosus, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, scleroderma, Wegener's granulomatosis, chronic active hepatitis, myasthenia gravis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, primary juvenile diabetes, dry eye associated with Sjögren's syndrome, uveitis posterior, and interstitial lung fibrosis.

Another aspect of the invention pertains to stimulating a T cell response by providing a costimulatory signal to a T cell. Delivery of a costimulatory signal, in conjunction with a primary activation signal, can generate a T cell response. In the method of the invention, a costimulatory signal is provided by contacting a T cell which has received a primary activation signal with an agent which stimulates production of D-3 phosphoinositides in the T cell. A T cell "response" is intended to encompass production of at least one lymphokine by the T cell (e.g., IL-2) and/or proliferation by the T cell. A primary activation signal can be delivered to a T cell by stimulating the T cell through the TCR/CD3 complex, for example by anti-CD3 antibodies or by an MHC/antigen complex, or by use of an agent which mimics this stimulation, for example a phorbol ester (e.g., PMA). The term "agent", as used herein, is intended to encompass chemicals and other pharmaceutical compounds which stimulate a costimulatory signal in a T cell without the requirement for an interaction betwen a T cell surface receptor and a costimulatory molecule. For example, the agent may act intracellularly to stimulate a signal associated with costimulation. In one embodiment, the agent is a non-proteinaceous compound. As the agent used in the method is intended to bypass the natural receptor:ligand costimulatory mechanism, the term agent is not intended to include a cell expressing a natural ligand of CD28/CTLA4 (e.g., expressing B7-1 and/or B7-2).

Preferably, production of D-3 phosphoinositides in the T cell is stimulated by contacting the T cell with an activator of PI3K. Activators of PI3K can be identified, for

example, by methods described below. Additional agents which stimulate one or more other intracellular signals associated with costimulation can be used in conjuction with an acitivator of D-3 phosphoinositide production. For example, the T cell can be contacted both with a first agent which stimulates PI3K activity and a second agent which stimulates protein tyrosine phosphorylation within the T cell. Protein tyrosine phosphorylation can be stimulated in the T cell, for example, by contacting a T cell with an activator of protein tyrosine kinases, such as pervanadate (see O'Shea, J.J. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10306-10310l; and Secrist, J.P. (1993) *J. Biol. Chem.* 268:5886-5893). The T cell can also be contacted with a first agent which stimulates PI3K and a second agent which stimulates protein serine phosphorylation within the T cell, for example by contacting a T cell with an activator of protein serine kinases. In another embodiment, the T cell is further contacted with a third agent which stimulates protein tyrosine phosphorylation. Alternatively, the T cell can be contacted both with an activator of PI3K and with an agent which inhibits the activity of a cellular protein tyrosine phosphatase, such as CD45, to increase the net amount of protein tyrosine phosphorylation in the T cell and/or with an agent which the activity of a cellular protein serine phosphatase. The method also encompasses stimulation of other intracellular signals associated with costimulation of a T cell, such as stimulation of phospholipase C activity and/or increases in intracellular calcium levels.

Another embodiment of the invention provides a method for stimulating a specific response to an antigen by an antigen-specific T cell. To stimulate a T cell response, an antigen-specific T cell is contacted with the antigen together with an agent which stimulates production of D-3 phosphoinositides in the T cell, thereby triggering a costimulatory signal in the T cell. Preferably, the agent which stimulates production of D-3 phosphoinositides in the T cell is an activator of PI3K. The T cell is contacted with the antigen in a form suitable for stimulating a primary activation signal in the T cell (e.g., through the TCR/CD3 complex), such as in conjuction with an MHC molecule. An antigen presenting cell (e.g., a B cell, macrophage, monocyte, dendritic cell, Langerhan cell, or other cell which can present antigen to a T cell) can be incubated with the T cell in the presence of the antigen (e.g., a soluble antigen). Alternatively, a cell expressing an antigen of interest can be incubated with the T cell. For example, a tumor cell expressing tumor-associated antigens can be incubated with a T cell together with an agent which induces an intracellular costimulatory signal to induce a tumor-specific response. Alternatively, a cell infected with a pathogen, e.g. a virus, which presents antigens of the pathogen can be incubated with a T cell in the presence of the agent. In addition to stimulating production of D-3 phosphoinositides in the T cell, the T cell can be contacted with one or more other agents which stimulate one or more additional intracellular signals associated with CD28 ligation, for example an activator of a protein tyrosine kinase, such as pervanadate or an activator of a serine kinase.

An agent which stimulates a CD28-associated intracellular signal in T cells, e.g., an activator of PI3K, can be administered to a subject *in vivo*, or alternatively, a T cell can be

obtained from a subject, stimulated *in vitro*, and readministered to the subject. The methods for stimulating T cell responses are useful in therapeutic situations where it is desirable to upregulate an immune response (e.g., induce a response or enhance an existing response). For example, the method can be used to enhance a T cell response against tumor-associated antigens. Tumor cells from a subject typically express tumor-associated antigens but may be unable to stimulate a costimulatory signal in T cells (e.g., because they lacks expression of costimulatory molecules). Thus, tumor cells can be contacted with T cells from the subject together with an agent which stimulates D-3 phosphoinositides in the T cell to trigger a costimulatory signal in the T cell. Alternatively, T cells can be stimulated as described herein to induce or enhance responsiveness to pathogenic agents, such as viruses (e.g., human immunodeficiency virus), bacteria, parasites and fungi. Additionally, the efficacy of vaccines against such pathogenic agents can be enhanced. For example, an agent which stimulates D-3 phosphoinositide production in T cells can be administered to a subject infected with a pathogenic agent or can be coadministered with a vaccine to enhance the responsiveness of T cells to antigens of the vaccine. Alternatively, T cells can be cultured *in vitro* which antigen presenting cells which express an antigen(s) from a pathogenic agent together with an agent which stimulates an intracellular signal associated with costimulation (e.g., an activator of PI3K).

Another application of the method for stimulating T cell responses pertains to patients who have impaired signal transduction through CD28 and/or other cell surface molecule(s) associated with costimulation (e.g., CTLA4). For example, a patient with idiopathic thrombocytopenia has been reported to exhibit defective CD28-mediated signal transduction, presumably due to a congenital defect (see Perez-Blas, M. et al. (1991) *Clin. Exp. Immunol.* 85:424-428). In patients having defective CD28 signalling ability, it may be possible to bypass the defect and restore CD28-dependent T cell activation by contacting T cells from the patient with one or more agents which stimulate intracellular signals generated upon normal CD28 ligation. For example, a patient having a defect resulting in reduced or a lack of D-3 phosphoinositide production upon CD28 ligation can be treated by contacting T cells from the patient with an agent which stimulates production of D-3 phosphoinositides in the T cells.

Another aspect of the invention pertains to screening assays for identifying inhibitors and activators of PI3K which can then be used to inhibit or stimulate, respectively, T cell responses. PI3K is a heterodimer consisting of a regulatory and a catalytic subunit. Two forms of the enzyme which preferentially use PtdIns(4,5)P_2 as a substrate and are inhibitable by wortmannin have been described (see Otsu, M et al. (1991) *Cell* 65:91-104; Hu, P. et al. (1993) *Mol. Cell. Biol.* 13:7677-7688; and Hiles, I.D. et al. (1992) *Cell* 70:419). Another form of the enzyme which preferentially uses PtdIns as a substrate and is not inhibitable by wortmannin has also been described (see Stephens. L. et al. (1994) *Curr. Biol.* 4:203-214). It will be appreciated that identification of specific inhibitors or activators of PI3K must be

specific to the appropriate intracellular form(s) of PI3K involved in costimulatory signals to avoid unwanted or adverse side effects. Thus, agents which specifically inhibit or activate a form(s) of PI3K involved in costimulation (e.g., a form which is also inhibitable by wortmannin) are preferable.

In one embodiment, a screening assay of the invention is based upon the ability of an inhibitor or activator of a PI3K to inhibit or stimulate, respectively, the production of at least one D-3 phosphoinositides in a T cell (preferably PtdIns(3,4,5)P_3). To identify an inhibitor of a PI3K, a T cell is stimulated through a cell surface receptor that binds a costimulatory molecule (i.e., a T cell which has received a costimulatory signal) in the presence and absence of a substance to be tested. Preferably, a T cell which expresses CD28 is used in the assay. Alternatively, a T cell which expresses CTLA4 can be used. A costimulatory signal can be stimulated in the T cell by contacting the T cell with a ligand for CD28 or CTLA4. Preferably, the ligand is a physiologic ligand, such as membrane-bound B7-1 or B7-2, rather than antibodies directed against the T cell surface receptor. A cell which naturally expresses B7-1 and/or B7-2 can be used or more preferably a cell (e.g., a CHO cell) which is transfected to express a costimulatory molecule is used. In the presence of an inhibitor of PI3K, stimulation of a T cell through a surface receptor which binds a costimulatory molecule (e.g., CD28) results in reduced production of D-3 phosphinositides in the T cell relative to stimulation in the absence of the inhibitor. Production of D-3 phosphoinositides in the T cell can be measured by any suitable method known in the art. For example, production of D-3 phosphoinositides in the T cell can be measured by high pressure liquid chromatography (as described in the Examples). Alternatively, D-3 phosphoinositide production can be assessed by thin layer chromatography, e.g. as described in Okada, T. et al. (1994) *J. Biol. Chem.* 269:3563-3567. D-3 phosphoinositides whose intracellular production can be assessed include PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Preferably, production of PtdIns(3,4,5)P_3 in the T cell is detected in the presence or absence of the substance to be tested.

To identify an activator of a PI3K, a T cell which expresses a cell surface receptor which binds a costimulatory molecule is contacted with a substance to be tested. An activator of a PI3K is identified based upon its ability to stimulate production of at least one D-3 phosphoinositides in a T cell (preferably PtdIns(3,4,5)P_3). Thus, in the presence of a PI3K activator, the amount of a D-3 phosphoinositide in the T cell is increased relative to the amount of the D-3 phosphoinositide in the T cell in the absence of the substance. Production of D-3 phosphoinositides (e.g., PtdIns(3)P, PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3) in the T cell can be assessed by standard methods, such as high pressure liquid chromatography or thin layer chromatography, as discussed above.

In another embodiment of the screening assays of the invention, the ability of a substance to directly inhibit or stimulate the activity of a PI3K isolated from a cell is assessed and then a substance identified as an inhibitor or activator of the PI3K is contacted with a T

cell to determine the ability of the substance to inhibit or stimulate a T cell response. For example, an isolated PI3K is incubated with a substrate (e.g. PtdIns(4,5)P_2) in the presence of a radiolabeled phosphate donor and a substance to be tested. An inhibitor of the kinase activity of the PI3K will cause reduced phosphorylation of the substrate (relative to phosphorylation in the absence of the inhibitor), whereas an activator will cause increased phosphorylation of the substrate (relative to phosphorylation in the absence of the activator). An inhibitor or activator so identified *in vitro* is then contacted with a T cell to determine the ability of the inhibitor or activator to inhibit or stimulate, respectively, a T cell response (e.g., IL-2 production).

The invention further pertains to a phosphotyrosine protein of approximately 67 kDa, which is associated with CD28 in Jurkat cells and peripheral blood T cells, as described in the Examples. This novel protein, termed 67K, appears to be different from itk/emt protein tyrosine kinase, lck and PI3K and is most likely involved in a signal transduction pathway associated with CD28. In a specific embodiment of the invention, the CD28 signal transduction pathway in which the 67K is involved is inhibited by blocking activation of the 67K, for example by blocking its phosphorylation. Alternatively, the signal transduction pathway associated with the 67K is blocked by inhibiting the interaction between the 67K and CD28. In another embodiment of the invention, the signal transduction pathway associated with the 67K is stimulated by activating the 67K or by stimulating interaction of the 67K with CD28. Activation or inhibition of the signal transduction pathway involving the 67K can be performed with any of the above described embodiments resulting in blocking of T cell responses, or alternatively in stimulating T cells responses.

Other Embodiments

Other cells types in addition to T cells have been described which express CD28 on their surface. These cell types include plasma cells (see Kozbor, D. et al. (1987) *J. Immunol.* 138:4128-4132) and bone marrow-derived mast cells. Stimulation of other $CD28^+$ cells types through CD28 may also lead to production of D-3 phosphoinositides in the cells and generation of specific cell responses. Inhibition or activation of D-3 phosphoinositide production in these cells, by the methods described herein, may also be useful for inhibiting or stimulating responses by other $CD28^+$ cell types.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLE 1: Ligation of CD28 Stimulates Production of D-3 Phosphoinositides

In this example, the production of D-3 phosphoinositides in $CD28^+$ Jurkat cells upon stimulation of the Jurkat cells through CD28 or CD3 was examined. Jurkat cells were labeled with carrier-free [^{32}P]-orthophosphate ([^{32}Pi]) as follows: Jurkat cells were washed 3 times in phosphate-free media (DMEM/RPMI) and incubated for 10 minutes at 37 °C for 10 minutes between washes. The cells were resuspended in 10 ml phosphate-free media containing 20 mM HEPES, pH 7.4 and 5 % dialysed fetal calf serum (dialysed overnight against saline). Carrier-free [^{32}Pi] was added to the cells (1 mCi/10 ml cells) and the cells were incubated at 37 °C for 4 to 6 hours. After labeling, cells were washed 3 times with phosphate-free media and resuspended in RPMI 1640 containing 20 mM HEPES.

In various experiments, aliquots of [^{32}Pi]-labeled Jurkat cells (0.15 ml; 2 x 10^7 cells) were stimulated with media alone, an anti-CD3 antibody (G19-4), an anti-CD28 antibody (9.3), untransfected CHO cells, or CHO cells transfected to express a CD28 ligand, either human B7-1 (CHO-B7-1) or B7-2 (CHO-B7-2). [obtained from Drs. G. Freeman and L. Nadler; CHO cells were transfected with a recombinant expression vector containing a cDNA encoding human B7-1, the sequence of which is disclosed in Freeman, G.J. et al. (1989) *J. Immunol.* 143:2714-2722, or a cDNA encoding human B7-2, the sequence of which is disclosed in Freeman, G.J. et al. (1993) *Science* 262:909-911, by standard techniques). For stimulation with CHO cells, Jurkat cells were incubated with 10^7 CHO cells and cell contact was achieved by low speed centrifugation in a microfuge for 5 seconds. At various time intervals following stimulation (ranging between about 1 and 25 minutes), the cells were lysed and phospholipids were extracted, deacylated and separated by anion exchange HPLC basically as described in Ward, S.G et al. (1992) *J. Immunol.* 22:45, with modifications as described below.

The incubations were terminated by addition of 750 ml $CHCl_3$/methanol/water (32.6%/65.3%/2.1% v/v/v). Once cell reactions were quenched, the samples were kept on ice during subsequent extractions. Phases were separated by addition of 725 ml $CHCl_3$ (containing 10 mg Folch lipids; e.g., from Sigma, Catalogue No. B1502) and 172 ml 2.4 M HCl, 5 mM tetrabutylammonium sulphate to each sample. The samples were vortexed and centrifuged for 5 minutes at 1000 rpm to separate phases. The lower phase was removed and added to a tube containing ½ volume of 1 M HCl, 25 mM Na_2EDTA, pH 7.0, 5 mM tetrabutylammonium sulphate. The samples were recentrifuged to separate phases, the bottom layer was removed and placed in a clean tube, and the sample was dried down in vacuo. Samples were deacylated by adding 1 ml methylamine reagent (40 % in water/methanol/n-butanol 4:4:1 v/v/v), vortexing and incubating at 53 °C for 40 minutes. Samples were placed on ice and then dried down in vacuo. The samples were resuspended in 0.5 ml sterile distilled water and vortexed to mix. The samples were extracted twice with 0.7 ml n-butanol:40-60% petroleum ether/ethyl formate (20:4:1 v/v/v). The bottom aqueous phase was dried in vacuo and stored at -70 °C prior to HPLC analysis.

HPLC was performed using a gradient based on buffers A (water)/B [1.25 M $(NH_4)_2HPO_4$] (adjusted to pH 3.8 with H_3PO_4 at 25 °C) and a Partisphere SAX column (commercially obtained from Whatman). Deacylated phospholipid samples were resuspended in 0.1 ml distilled water and injected onto the column. The eluate was fed into a Canberra Packard A-500 Flo-One on-line *beta*-radiodetector, where it was mixed with three parts Flo-Scint IV scintillation cocktail and the results were analyzed on the Flo-One data program (Radiomatic, USA). Eluted peaks were compared to retention times for standards prepared from [^{3}H]PtdIns, [^{3}H]PtdIns(4)P and [^{3}H]PtdIns(4,5)P_2 (commercially obtained from Amersham International). Standard [^{32}P]PtdIns(3)P, [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PtdIns(3,4,5)P_3 were prepared by incubating isolated phosphatidylinositol 3-kinase with PtdIns (commercially obtained from Sigma) as described in Ward, S. G. et al. (1992) *J. Biol. Chem.* 267:23862.

The production of PtdIns(3,4,5)P_3 in Jurkat cells following stimulation with anti-CD3 or anti-CD28 antibodies for 1-5 minutes is shown in Figure 1. The results demonstrate that while stimulation through either CD3 or CD28 induces PtdIns(3,4,5)P_3 production, the induction kinetics for the two pathways are distinct. Upon CD3 stimulation, PI3K activation (as assessed by PtdIns(3,4,5)P_3 production) increases early (i.e., within 2 minutes) and is transient (i.e., returns to baseline by 5 minutes). In contrast, PI3K activation induced by CD28 stimulation is delayed compared to CD3 stimulation (i.e., is not maximal until 5 minutes or later) and persists longer than that induced by CD3 stimulation. These results indicate that distinct mechanisms are involved in PI3K activation mediated by either CD3 or CD28 ligation.

The production of PtdIns(3,4,5)P_3 in Jurkat cells following stimulation with CHO-B7-1 or CHO-B7-2 cells for 0-20 minutes is shown in Figure 2. The results indicate that stimulation of CD28 with either B7-1 or B7-2 induces potent activation of PI3K (as assessed by PtdIns(3,4,5)P_3 production). The induction kinetics are slightly different for the two CD28 ligands: B7-1 stimulates activation earlier than B7-2, although both plateau to the same level. Stimulation of PtdIns(3,4,5)P_3 production by either B7-1 and B7-2 is very strong and persistent (ie., continues for more than 20 minutes).

This example demonstrates that stimulation of T cells through CD28, either by its natural ligands B7-1 and B7-2 or by an anti-CD28 antibody, induces the production of D-3 phosphoinositides within T cells, indicating activation of PI3K upon CD28 ligation. In addition, this example demonstrates that CD28 shares in common B7-1 and B7-2 as physiological ligands, since Jurkat cells are CD28$^+$ but CTLA4$^-$ and cannot be induced to express CTLA4 (as shown in Lindsten, T. (1993) J. Immunol. 151:3489-3499). Thus, CTLA4 apparently is not required for B7-induced signal transduction and both B7-1 and B7-2 are physiologic ligands for CD28.

EXAMPLE 2: A Phosphatidylinositol 3-Kinase Inhibitor Can Inhibit Production of D-3 Phosphoinositides Induced by CD28 Ligation

In this example, the effect of an inhibitor of phosphatidylinositol 3-kinase on CD28-mediated production of D-3 phosphoinositides within Jurkat cells was examined. Jurkat cells were labeled with orthophosphate and stimulated with CHO cells transfected to express B7-2, as described in Example 1. Additionally, during stimulation, the cells were incubated in the presence of various concentrations (0-100 mM) of the fungal metabolite wortmannin, which is an inhibitor of phosphatidylinositol 3-kinase. Wortmannin was obtained commercially from Sigma Chemical Co. and stored as a 10 mM solution in DMSO at -40 °C. It was diluted in medium immediately before addition to cells in culture. Following stimulation in the presence or absence of wortmannin, the amount of PtdIns(3,4,5)P_3 produced in the cells was measured by HPLC, as described in Example 1. The results are shown in Figure 3, wherein the amount of PtdIns(3,4,5)P_3 detected in wortmannin-treated Jurkat cells upon stimulation with CHO-B7-2 is plotted graphically as a percentage of the amount of PtdIns(3,4,5)P_3 detected in untreated Jurkat cells stimulated with CHO-B7-2. The results demonstrate that treatment of Jurkat cells with increasing concentrations of wortmannin decreases the amount of D-3 phosphoinositides produced in the cells upon ligation of CD28 with B7-2. Accordingly, this example demonstrates that the generation of D-3 phosphoinositides intracellularly as a result of stimulation of T cells through CD28 can be inhibited by inhibiting the activity of phosphatidylinositol 3-kinase within the T cells.

EXAMPLE 3: Effect of Pharmacological Inhibitors on CD28-Mediated Calcium Flux

In this example, the effect of pharmacological inhibitors on calcium flux in Jurkat cells induced by anti-CD28 antibodies was examined. The pharmacological inhibitors studied were wortmannin, which inhibits the activity of PI3K, and herbimycin A, which inhibits the activity of protein tyrosine kinases. Jurkat cells were stimulated with an anti-CD28 antibody, either in medium alone or in the presence of wortmannin or herbimycin A, and the mean calcium concentration (nM) in the cells was measured over several minutes following stimulation. As illustrated in Figure 4, herbimycin A was capable of inhibiting CD28 antibody-induced calcium flux. In contrast, wortmannin was unable to inhibit CD28-antibody induced calcium flux. These results indicate that the effects of wortmannin on T cells are not mediated by interfering with calcium flux. Furthermore, given results described below in Example 5 showing that wortmannin inhibits costimulation as measured by IL-2 production induced by B7-1 or B7-2, this data indicates that measurement of CD28-induced calcium elevation is likely to be a misleading read-out for assessing compounds that specifically induce T cell unresponsiveness (i.e., anergy) or costimulation.

EXAMPLE 4: Induction of Calcium Flux by Natural CD28 Ligands

In this example, adhesion of CHO-B7-1 or CHO-B7-2 to Jurkat cells and induction of calcium mobilization in Jurkat cells in response to stimulation with CHO-B7-1 and B7-2 were examined. A flow cytometic cell calcium-conjugate assay, as described in Abe, R. et al. (1992) *J. Exp. Med.* 176:459-468, was used. In the cell conjugate assay, T cells are loaded with the calcium sensitive fluorescent probe indo-1 (which generates blue and green signals). CHO cells transfected with a control plasmid (CHO-neo) or CHO cells transfected to express B7-1 or B7-2 are loaded with the tracer dye DilC22(3) (which generates red signals; obtained commercially from Molecular Probes). Jurkat cell-CHO cell conjugates are analyzed by flow cytometry. Conjugates consisting of T cells and CHO cells can be measured by gating on red signals and calcium levels can be measured in the T cells by gating on the blue and green signals. The results are displayed as a series of two paramater dot plots, shown in Figure 5. Calcium (indo-1 ratio) is on the Y axis and cell conjugates (red tracer) is on the X axis. Cells in the upper right quadrant represent Jurkat cells having high levels of calcium conjugated to CHO cells. Cells in the lower right quadrant represent Jurkat cells having normal calcium levels conjugated to CHO cells. Cells in the upper and lower left quadrants represent non-conjugated Jurkat cells having high or low levels of calcium, respectively.

The data indicates that both B7-1 and B7-2 can mediate adhesion to Jurkat cells. However, both ligands are poor at causing increases in calcium mobilization. Therefore, B7-1 and B7-2 are much more efficient at inducing PI3K activation (see Example 1) than calcium mobilization. In contrast, anti-CD28 antibodies are capable of stimulating both PI3K activation (see Example 1) and calcium flux (see Example 3). Thus, it appears that there are differences in the intracellular signals generated through CD28 ligation, depending upon whether natural ligands (e.g., B7-1 or B7-2) or antibodies are used for stimulation. It has previously been described (Nunes, J. et al. (1993) Int. Immunol. 5:311-315) that CD28 antibodies can have multiple and distinct effects on biochemical aspects of T cell signal transduction and activation (e.g., IL-2 production). These observations further indicate that it was not possible to predict the biochemical effects of natural ligands of CD28 (i.e., membrane-bound B7-1 and B7-2) on production of D-3 metabolites, as described herein, based upon extrapolation from previous results with CD28 antibodies.

EXAMPLE 5: A Phosphatidylinositol 3-Kinase Inhibitor Can Inhibit Production of Interleukin-2 Induced by CD28 Ligation

In this example, the effect of an inhibitor of phosphatidylinositol 3-kinase on CD28-dependent production of interleukin-2 by T cells was examined. In a first series of experiments, the effect of T cell stimulation through CD28, in conjunction with stimulation through CD3, on IL-2 production was assessed in the absence of wortmannin. Highly

purified human peripheral blood T cells were incubated for 24 hours with an immobilized anti-CD3 antibody (OKT3) alone or together with either an anti-CD28 antibody (9.3) or mitomycin C-treated CHO cells, either untransfected or transfected to express B7-1 or B7-2. Increasing numbers of CHO cells were tested (0.5×10^6 to 4×10^6). After culture for 24 hours, the culture supernatants were assayed for IL-2 production by ELISA by standard techniques. As shown in Figure 6, cells incubated in medium alone, OKT3 alone, or OKT3 together with untransfected CHO cells did not produce IL-2. In contrast, culture of the cells with OKT3 together with CHO cells expressing B7-1 or B7-2 stimulated IL-2 production in a dose dependent manner. Culture with OKT3 and 9.3 antibodies also stimulated IL-2 production. These results confirm that CD28 ligation, such as by B7-1 or B7-2 stimulation, can provide a costimulatory signal for lymphokine production.

In a next series of experiments, resting human T cells were stimulated with either: 1)immobilized OKT3 + CHO-B7-1, 2) immobilized OKT3 + CHO-B7-2, 3) immobilized OKT3 + CHO-B7-1+B7-2, 4) PMA + CHO-B7-1 or 5) PMA + B7-2. Stimulation of T cells was performed in media alone or in media containing wortmannin at concentrations between 1 nM and 1 mM. Twenty-four hours following culture, the supernatants were assayed for IL-2 production by ELISA. The results are shown in Figures 7A and 7B. The results indicate that wortmannin can inhibit IL-2 production stimulated by either B7-1 or B7-2 in conjunction with CD3 stimulation. Wortmannin-mediated inhibition of IL-2 production was dose dependent. The ID_{50} for inhibition of B7-2-mediated stimulation was approximately 10 nM. The ID_{50} for inhibition of B7-1-mediated stimulation was between 10 and 100 nM. These doses of wortmannin are not generally toxic to the cells and do not inhibit IL-2 production by a non-specific mechanism, as evidenced by the fact that IL-2 production stimulated by PMA together with membrane-bound B7-1 or B7-2 was not inhibited by wortmannin at concentrations as high as 1 mM. This example demonstrates that T cell activation, as assessed by production of IL-2 in response to stimulation through the TCR/CD3 complex and CD28, can be inhibited by treatment of the T cell with an agent which inhibits the activity of phosphatidylinositol 3-kinase within the T cell.

EXAMPLE 6: Differential effect of herbimycin on PI 3K activation of Jurkat cells stimulated with anti-CD28 or the natural ligands of CD28

To assess the means of PI 3-kinase activation, Jurkat T cells (20×10^6 cells) were stimulated with anti-CD28 mAb 9.3 (10 μg) or with CHO cells expressing B7-1 or B7-2 (10×10^6 cells). The cells were labeled with ^{32}P orthophosphate, as described in Example 1, and either preincubated in medium containing vehicle diluent or in 3 μM herbimycin A. After 5 minutes the cells were lysed and PI 3-kinase assessed by measurement of PtdIns(3,4,5)P3 by HPLC as described in Example 1.

The results are presented in Figure 8. Production of PtdIns(3,4,5)P3 by Jurkat cells stimulated with 9.3 antibody was prevented by herbimycin pretreatment. Unexpectedly, B7-1

and B7-2 induced PI 3-kinase activation as measured by the production of PtdIns(3,4,5)P3 was resistant to herbimycin pretreatment. These results indicate that a herbimycin independent pathway for activation of PI 3-kinase by CD28 exists, and since herbimycin has been shown to inhibit a variety of src family kinases, that CD28 can activate PI 3-kinase independent of src family protein tyrosine kinases.

To further investigate this dichotomy, 20 x 10^6 Jurkat cells were stimulated with 9.3 mAb (10μg) or with 10 x 10^6 CHO-B7-1 cells. The cells were either preincubated with herbimycin A 3μM or in vehicle. The CD28 mAb was further crosslinked with goat anti-mouse IgG and the cells lysed after 5 to 10 min. PI 3-kinase activation was assessed by thin layer chromatography using an *in vitro* lipid kinase assay, as described in Ward, S.G. et al. (1995) *Eur. J. Immunol.* 25:526-532.

The results are represented in Figure 9. Both the anti-CD28 9.3 mAb and CHO-B7 cells induced PI 3-kinase activity as evident from a comparison of the control lanes to the 10 minutes 9.3 mAb lanes and the 5 minutes B7 lanes. Again, the polar kinase products of PI were inhibited by herbimycin after 9.3 mAb stimulation and resistant to herbimycin after B7 stimulation.

Thus, herbimycin blocks activation of PI 3K in Jurkat cells stimulated with anti-CD28, but not in Jurkat cells stimulated with CHO-B7-1 or CHO-B7-2 cells, indicating the existence of herbimycin independent pathway for activation of PI 3-kinase by CD28.

EXAMPLE 7: Differential effects of herbimycin on the secretion of IL-2 by T cells.

To further determine the signal transduction pathways that are involved with CD28 receptor ligation, $CD28^+$T cells (prepared by negative selection of peripheral blood T cells) were cultured overnight in various concentrations of herbimycin (from 0.1 to 3 μM) and stimulated with PMA at 3 ng/ml only or with mAb 9.3 in solution at 1 μg/ml, mAb 9.3 coated beads, ionomycin at 0.08 μg/ml, CHO-B7-1, or with CHO-B7-2 cells. Supernatants were harvested after 24 hours and analyzed for IL-2 content by ELISA.

The results are represented in Figure 10 panel A. The results indicate that PMA plus 9.3 mAb induced IL-2 secretion was sensitive to herbimycin with an ID_{50} of about 0.2 μM. Similarly, IL-2 secretion induced by PMA plus CHO-B7-1 cells was also sensitive to herbimycin. In contrast, IL-2 secretion was quite resistant when cells were stimulated with PMA plus ionomycin, with an ID_{50} of >3 μM. To further test the mechanism of inhibition by herbimycin, cells were also stimulated in the presence or absence of ionomycin. IL-2 secretion in cells stimulated with PMA plus ionomycin plus 9.3 mAb becomes quite resistant to herbimycin, even at 3 μM herbimycin A.

To further test this finding of herbimycin sensitive and resistant IL-2 secretion, T cells were stimulated with CD3 antibody OKT3 immobilized on beads with either CHO-B7-1 or

CHO-B7-2 cells, or with 9.3 mAb in the presence or absence of ionomycin. Supernatants were collected after 24 hours and the amount of IL-2 determined by ELISA.

The results are represented in Figure 10 panel B. Contrarily to IL-2 secretion resulting from stimulation of T cells with PMA and B7-1 or B7-2, which was found to be sensitive to herbimycin, IL-2 secretion was found to be quite resistant to herbimycin when PMA was replaced with anti-CD3. These results indicate that CD28 can trigger herbimycin resistant IL-2 secretion, depending on the context of the signal. When T cells were activated in a manner that does not trigger calcium signals (i.e., PMA plus anti-CD28) then IL-2 secretion was herbimycin sensitive. In contrast when T cells were activated in the presence of a calcium signal (i.e., PMA plus ionomycin plus B7 or anti-CD28), then the signal was resistant to herbimycin. This indicates that either CD28 does not require an essential signal provided by herbimycin sensitive tyrosine kinases or alternatively, that, a calcium signal may be able to bypass a requirement for an essential tyrosine kinase cascade that is triggered by CD28.

EXAMPLE 8: Identification of a 67 kDa tyrosyl phoshoprotein associated with CD28.

To date, the only reported proteins to display physical association with CD28 are PI 3-kinase (Truitt, K.E., et al. (1994) *J. Exp. Med.* 179:1071-1076) and the src family kinase lck (August, A. and Dupont, B. (1994) *Biochem. Biophys. Res. Commun.* 199:1466-1473). To further study CD28 mediated signal transduction, a search was initiated in Jurkat cells and T cells for proteins that are physically associated with CD28. Cells were stimulated with 9.3 mAb for various times, lysed in NP-40 detergent, and immunoprecipitation of CD28 done with Staphiloccocal protein A. The immunoprecipitates were washed, eluted, and separated by SDS-PAGE under reducing conditions. After transfer, immunoblots were done using antiphosphotyrosine Ab 4G10 coupled to horse radish peroxidase. Tyrosine phosphorylated proteins were identified by chemiluminescence using ECL.

The results are presented in Figure 11, which represents an autoradiography of an immunoblot. A band with relative migration of ~67 kDa was identified in the CD28 immunoprecipitations. This band corresponding to the phosphoprotein appears rapidly after CD28 stimulation, within 3 minutes in Jurkat cells. A similar or identical protein has been identified in T cell blasts. This protein does not appear to be the itk/emt protein tyrosine kinase because antibodies to itk do not reveal itk in CD28 immunoprecipitates. Furthermore, the molecular weight of this CD28 associated protein, is not consistent with lck or PI 3-kinase. Therefore, this is a novel CD28 associated protein.

Thus, the results show that a novel phosphotyrosine protein of approximately 67 kDa is associated with CD28 in Jurkat cells and in T cell blasts stimulated through CD28.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

5

CLAIMS

1. A method for inhibiting a response by a T cell expressing a cell surface receptor which binds a costimulatory molecule, comprising contacting the T cell with an agent which inhibits production of D-3 phosphoinositides in the T cell.

2. The method of claim 1, wherein the agent is an inhibitor of phosphatidylinositol 3-kinase.

3. The method of claim 2, wherein the inhibitor of phosphatidylinositol 3-kinase is selected from a group consisting of wortmannin, quercetin and LY294002, and derivatives or analogues thereof.

4. The method of claim 1, wherein the response by the T cell comprises production of at least one lymphokine.

5. The method of claim 4, wherein the lymphokine is interleukin-2.

6. The method of claim 1, wherein the response by the T cell comprises proliferation.

7. The method of claim 1, further comprising contacting the T cell with a second agent which inhibits protein tyrosine phosphorylation in the T cell.

8. The method of claim 7, wherein the second agent is an inhibitor of a protein tyrosine kinase.

9. The method of claim 8, wherein the inhibitor of a protein tyrosine kinase is herbimycin A or a derivative or analogue thereof.

10. The method of claim 7, wherein the second agent is a tyrosine phosphatase or an activator of a tyrosine phosphatase.

11. The method of claim 10, wherein the tyrosine phosphatase is a cellular tyrosine phosphatase.

12. The method of claim 11, wherein the cellular tyrosine phosphatase is CD45 or Hcph.

13. The method of claim 12, wherein the second agent is a molecule which binds to and activates CD45.

14. The method of claim 13, wherein the second agent is an anti-CD45 antibody, or fragment thereof.

15. A method for inducing unresponsiveness to an antigen in a T cell expressing a cell surface receptor which binds a costimulatory molecule, comprising contacting the T cell with the antigen and an agent which inhibits production of D-3 phosphoinositides in the T cell.

16. The method of claim 15, wherein the agent is an inhibitor of phosphatidylinositol 3-kinase.

17. The method of claim 16, wherein the inhibitor of phosphatidylinositol 3-kinase is selected from a group consisting of wortmannin, quercetin and LY294002, and derivatives or analogues thereof.

18. The method of claim 15, wherein the antigen is an alloantigen.

19. The method of claim 15, wherein the antigen is an autoantigen.

20. The method of claim 15, wherein the T cell is contacted with the antigen and the agent *in vitro* and the method further comprises administering the T cell to a subject.

21. A method of claim 20, wherein the antigen is on a surface of an allogeneic or xenogeneic cell and the subject is a recipient of an allogeneic or xenogeneic cell.

22. A method of claim 20, wherein the subject is suffering from an autoimmune disease or a disorder associated with an inappropriate or abnormal immune response.

23. A method for stimulating a response by a T cell which has received a primary activation signal and expresses a surface receptor that binds a costimulatory molecule, comprising contacting the T cell with an agent which stimulates production of D-3 phosphoinositides in the T cell.

24. The method of claim 23, wherein the agent is an activator of phosphatidylinositol 3-kinase.

25. The method of claim 23, wherein the response by the T cell comprises production of at least one lymphokine.

26. The method of claim 25, wherein the lymphokine is interleukin-2.

27. The method of claim 23, wherein the response by the T cell comprises proliferation.

28. The method of claim 23, further comprising contacting the T cell with a second agent which stimulates protein tyrosine phosphorylation in the T cell.

29. The method of claim 28, wherein the second agent is an activator of a protein tyrosine kinase.

30. The method of claim 28, wherein the second agent is an inhibitor of a cellular tyrosine phosphatase.

31. The method of claim 30, wherein the cellular tyrosine phosphatase is CD45.

32. A method for stimulating a response to an antigen by a T cell expressing a cell surface receptor which binds a costimulatory molecule comprising contacting the T cell with the antigen and an agent which stimulates production of D-3 phosphoinositides in the T cell.

33. The method of claim 32, wherein the agent is an activator of phosphatidylinositol 3-kinase.

34. The method of claim 32, wherein the antigen is a tumor-associated antigen.

35. The method of claim 32, wherein the antigen is from a pathogen selected from the group consisting of a bacteria, a virus, a fungus and a parasite.

36. The method of claim 32, wherein the T cell is contacted with the antigen and the agent *in vitro* and the method further comprises administering the T cell to a subject.

37. A method of claim 36, wherein the antigen is expressed by a tumor cell present in the subject.

38. A method of claim 36, wherein the antigen is expressed by a pathogen present in the subject.

39. A method for identifying an inhibitor of a phosphatidylinositol 3-kinase comprising:

a) providing a T cell which expresses a receptor that binds a costimulatory molecule;

b) stimulating an intracellular signal transduction pathway in the T cell associated with ligation of the receptor in the presence of an agent to be tested; and

c) determining an amount of at least one D-3 phosphoinositide produced in the T cell,

wherein a reduced amount of at least one D-3 phosphoinositide produced in the T cell in the presence of the agent relative to an amount produced in the T cell in the absence of the agent indicates that the agent is an inhibitor of a phosphatidylinositol 3-kinase.

40. The method of claim 39, wherein the receptor is CD28.

41. The method of claim 40, wherein the T cell is contacted with a ligand for CD28.

42. The method of claim 40, wherein the ligand for CD28 is a membrane-bound form of a B lymphocyte activation antigen selected from the group consisting of B7-1 and B7-2.

43. The method of claim 39, wherein production of at least one D-3 phosphoinositide in the T cell is measured by high pressure liquid chromatography.

44. A method for identifying an activator of phosphatidylinositol 3-kinase comprising:

a) contacting a T cell which expresses a receptor that binds a costimulatory molecule with an agent to be tested; and

b) determining an amount of at least one D-3 phosphoinositide produced in the T cell,

wherein an increased amount of at least one D-3 phosphoinositide produced in the T cell in the presence of the agent relative to an amount produced in the T cell in the absence of the agent indicates that the agent is an activator of a phosphatidylinositol 3-kinase.

45. The method of claim 44, wherein production of at least one D-3 phosphoinositide in the T cell is measured by high pressure liquid chromatography.

46. The method of claim 1, further comprising contacting the T cell with a second agent which inhibits protein serine phosphorylation in the T cell.

47. The method of claim 7, further comprising contacting the T cell with a third agent which inhibits protein serine phosphorylation in the T cell.

48. The method of claim 15, further comprising contacting the T cell with a second agent which inhibits protein tyrosine phosphorylation in the T cell.

49. The method of claim 15, further comprising contacting the T cell with a second agent which inhibits protein serine phosphorylation in the T cell.

50. The method of claim 48, further comprising contacting the T cell with a third agent which inhibits protein serine phosphorylation in the T cell.

51. The method of claim 23, further comprising contacting the T cell with a second agent which stimulates protein serine phosphorylation in the T cell.

52. The method of claim 28, further comprising contacting the T cell with a third agent which stimulates protein serine phosphorylation in the T cell.

53. The method of claim 32, further comprising contacting the T cell with a second agent which stimulates protein tyrosine phosphorylation in the T cell.

54. The method of claim 32, further comprising contacting the T cell with a second agent which stimulates protein serine phosphorylation in the T cell.

55. The method of claim 53, further comprising contacting the T cell with a third agent which stimulates protein serine phosphorylation in the T cell.

56. An isolated protein of approximately 67 kDa which is associated with CD28 and tyrosine phosphorylated in activated T cells.

FIG. 1

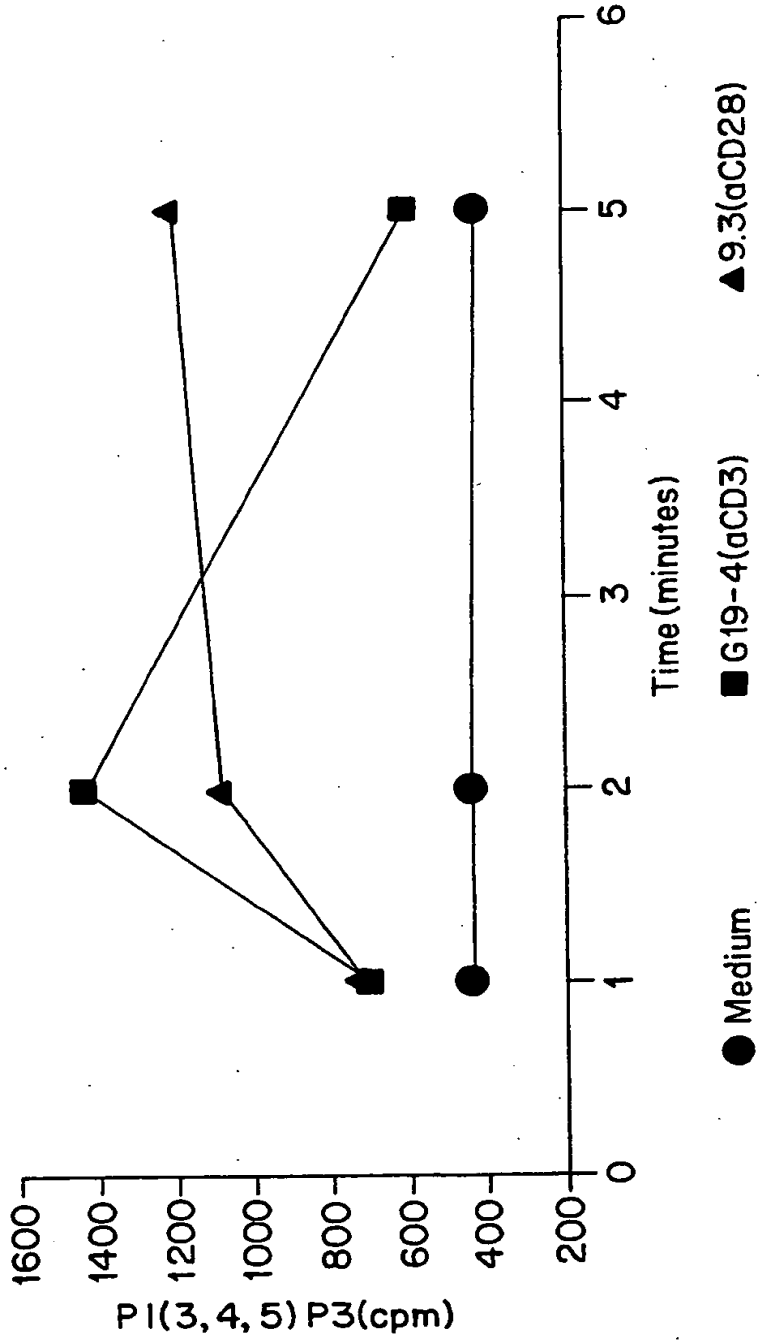
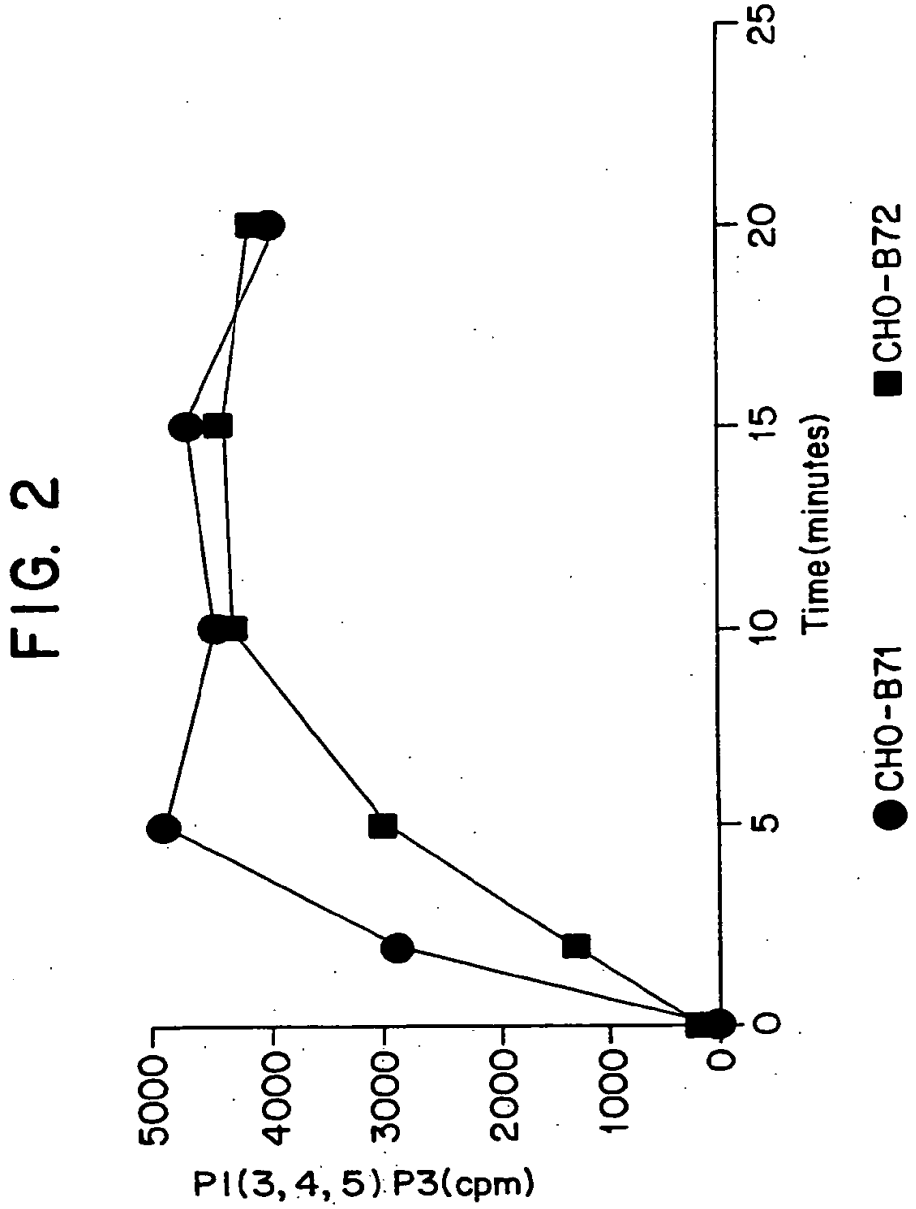


FIG. 2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4000
3000
2000
1000
0
PI(3, 4, 5) P3(cpm)
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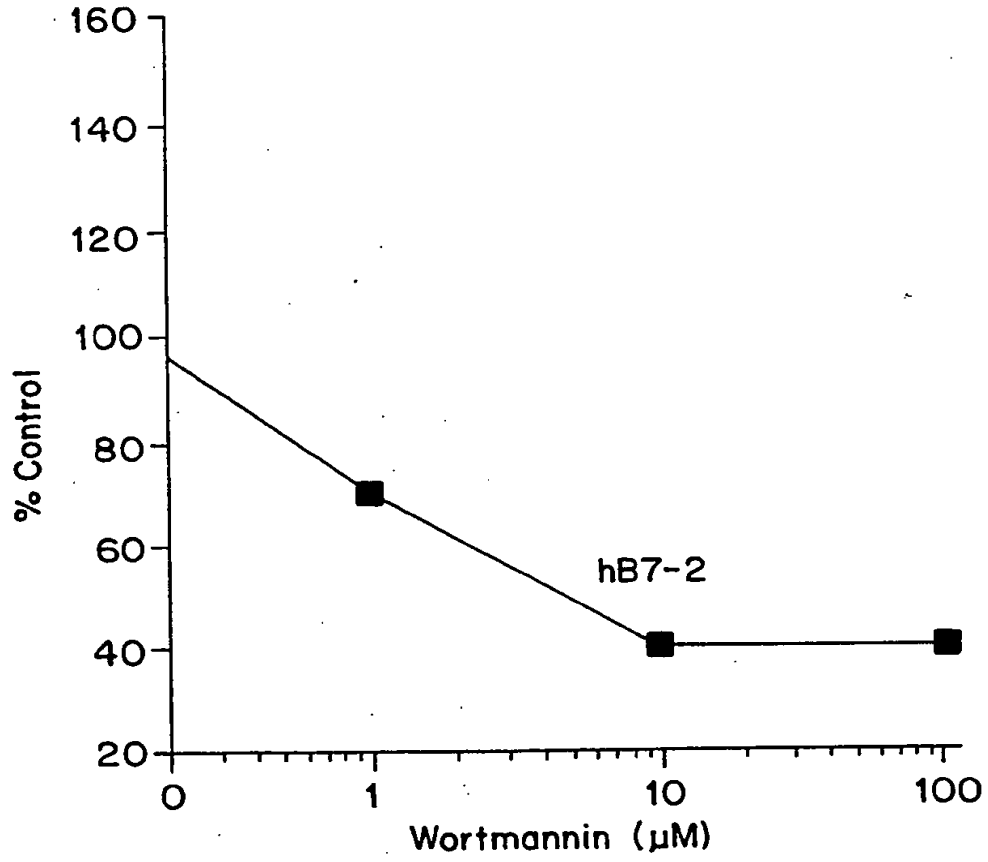
5
10
15
20
25
Time(minutes)
CHO-B71
CHO-B72

FIG. 3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140
120
100
80
60
40
20
% Control
hB7-2
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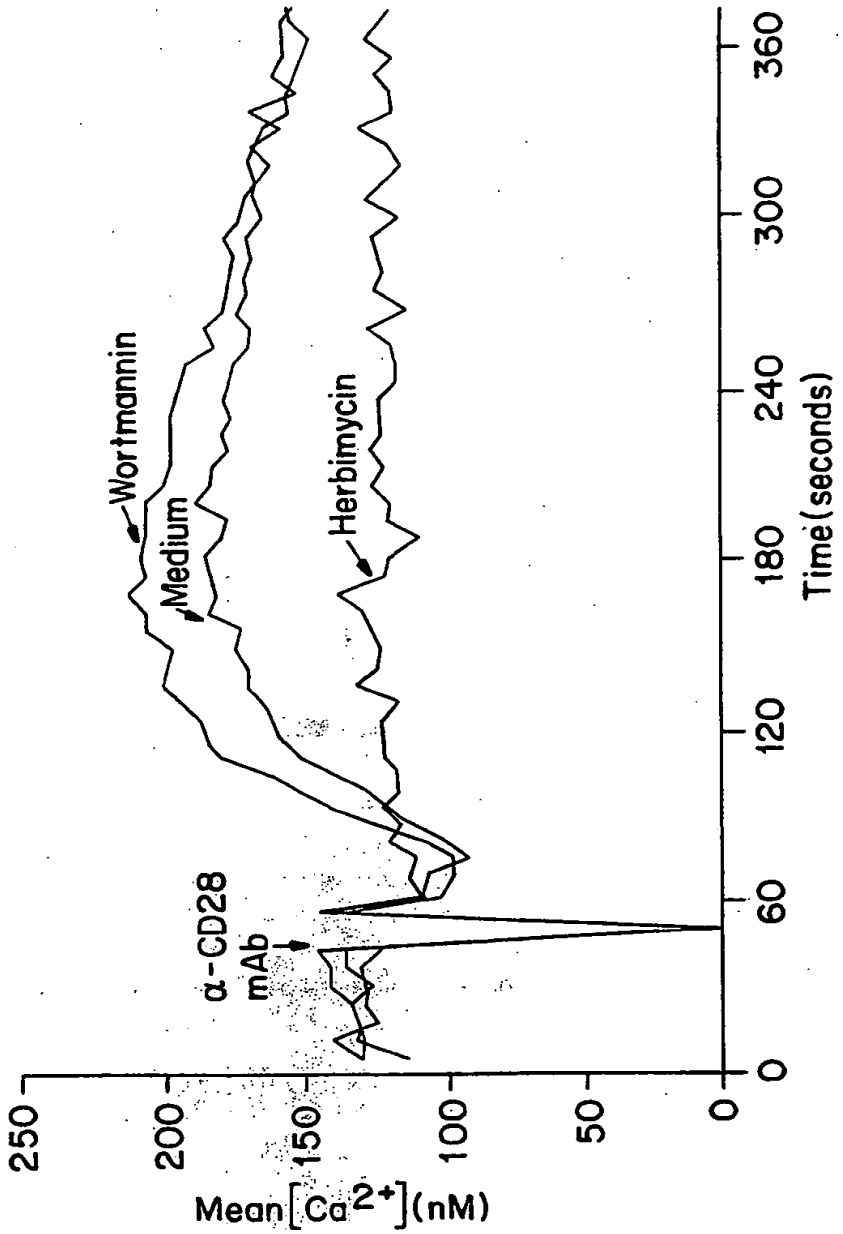
1
10
100
Wortmannin (μM)

FIG. 4


Wortmannin
Medium
Herbimycin
α-CD28 mAb
Mean [Ca2+] (nM)
Time (seconds)
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200
150
100
50
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0
60
120
180
240
300
360

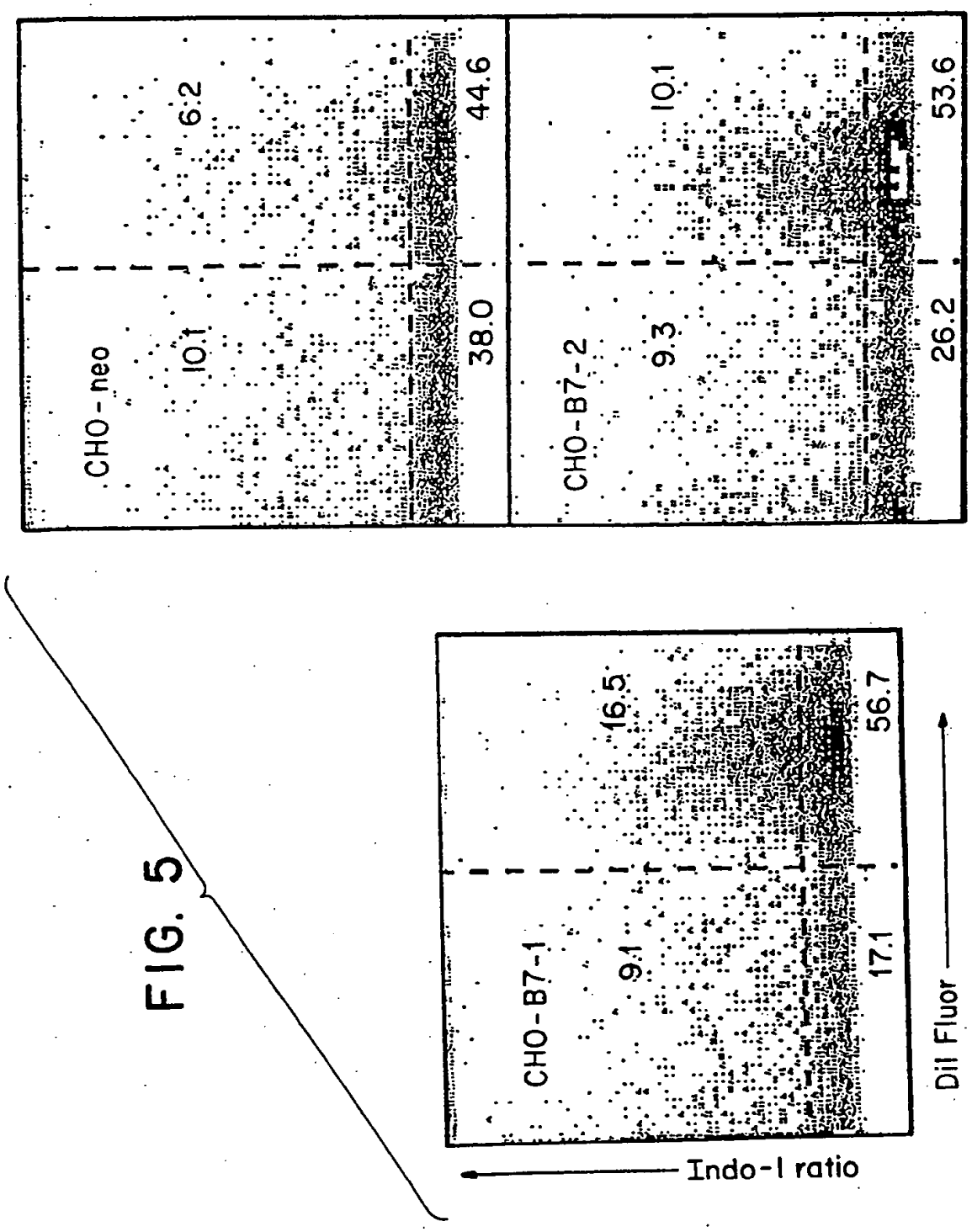
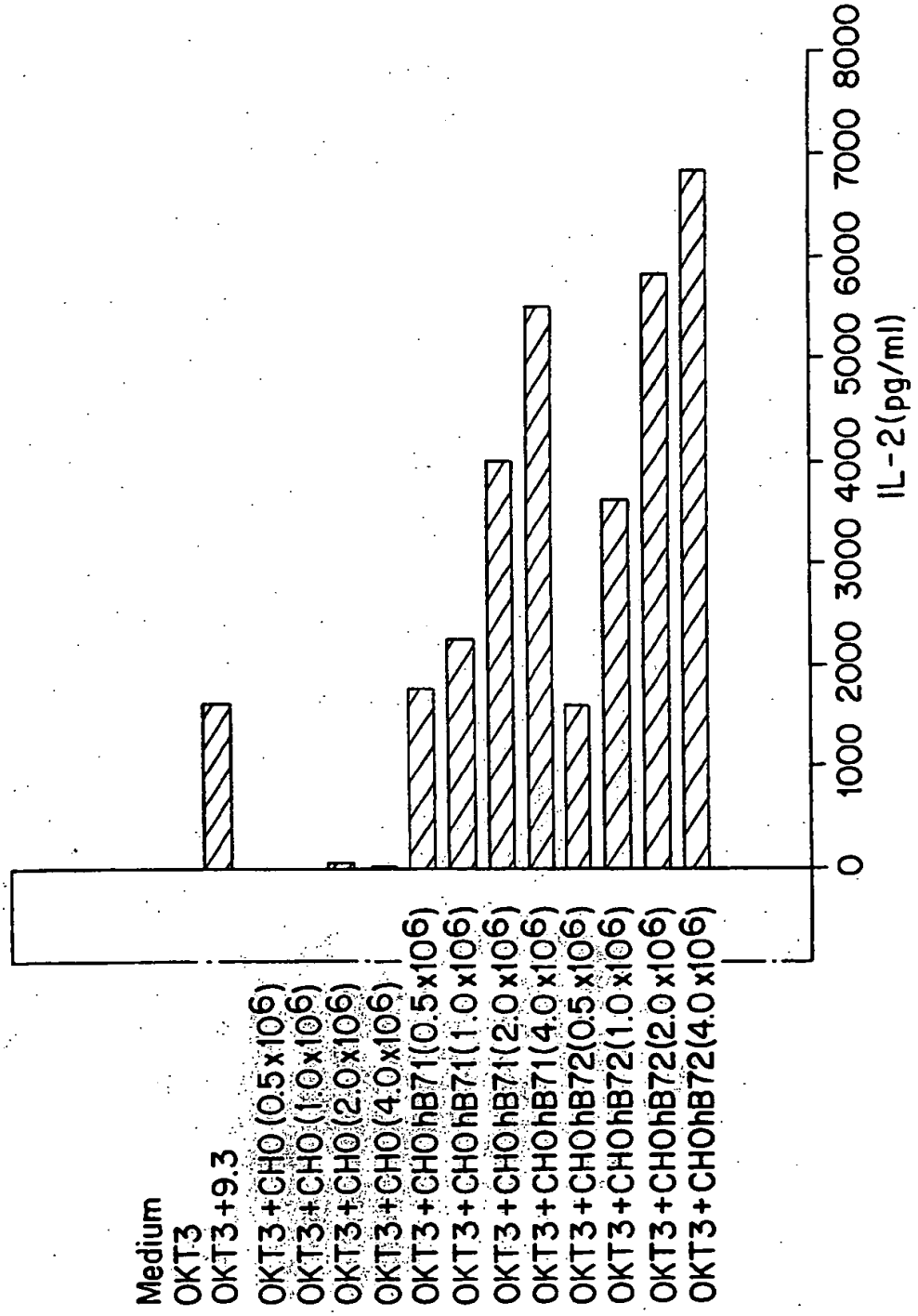


FIG. 5

FIG. 6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1000
2000
3000
4000
5000
6000
7000
8000
Medium
OKT3
OKT3+9.3
OKT3+CHO(0.5x106)
OKT3+CHO(1.0x106)
OKT3+CHO(2.0x106)
OKT3+CHO(4.0x106)
OKT3+CHOhB71(0.5x106)
OKT3+CHOhB71(1.0x106)
OKT3+CHOhB71(2.0x106)
OKT3+CHOhB71(4.0x106)
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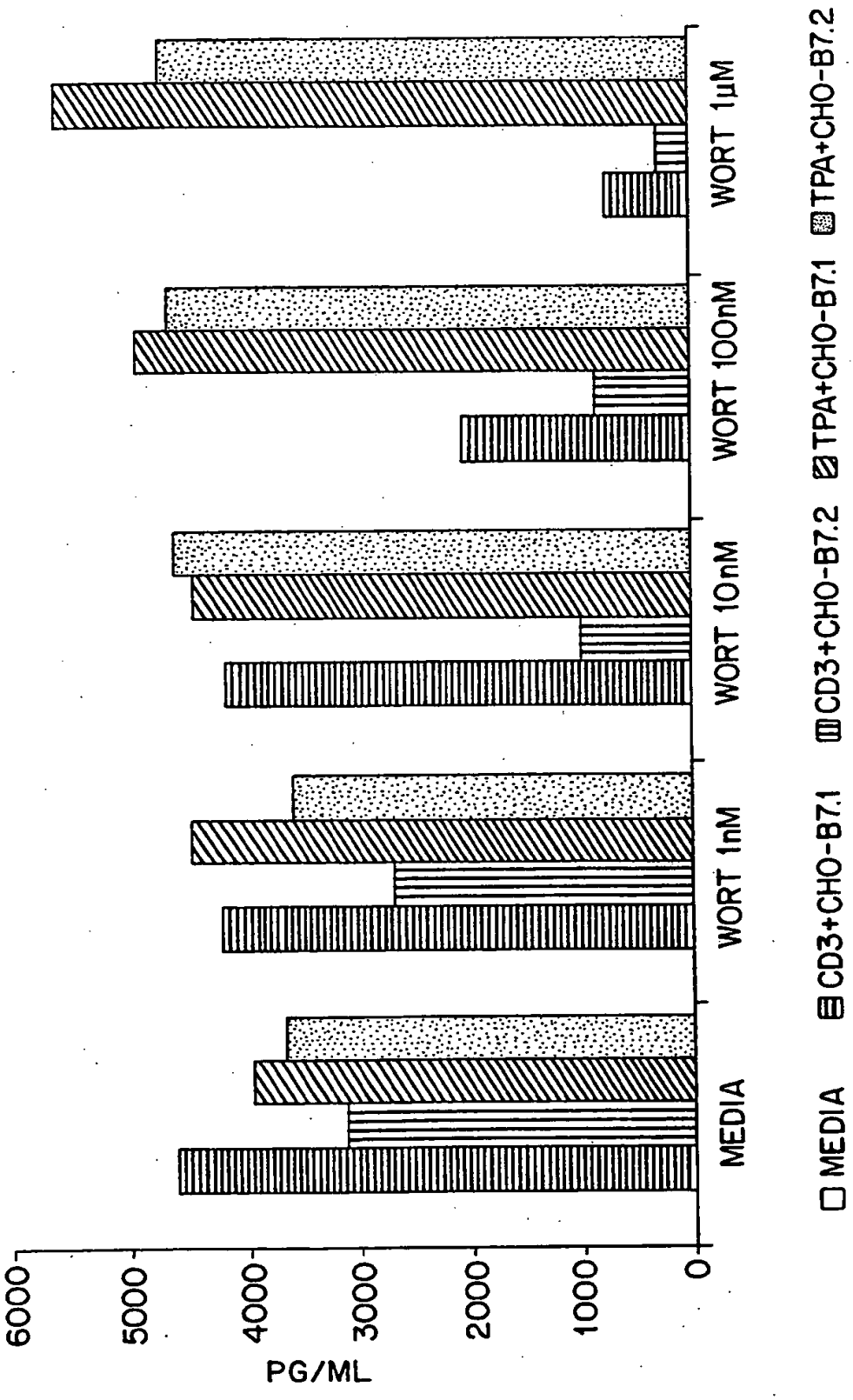
FIG. 7A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5000
4000
3000
2000
1000
0
PG/ML
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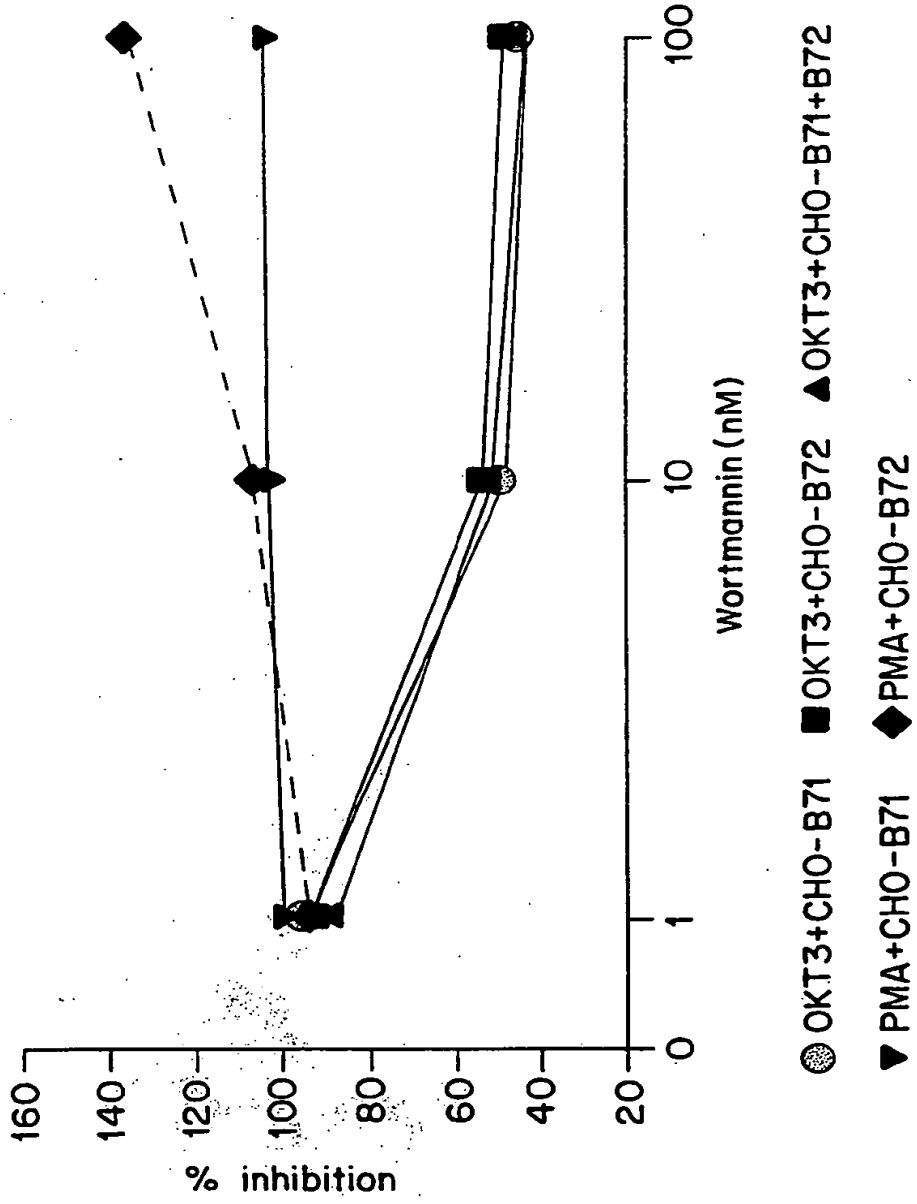
WORT 10nM
WORT 100nM
WORT 1μM
MEDIA
CD3+CHO-B7.1
CD3+CHO-B7.2
TPA+CHO-B7.1
TPA+CHO-B7.2

FIG. 7B


% inhibition
160
140
120
100
80
60
40
20
0
1
10
100
Wortmannin (nM)
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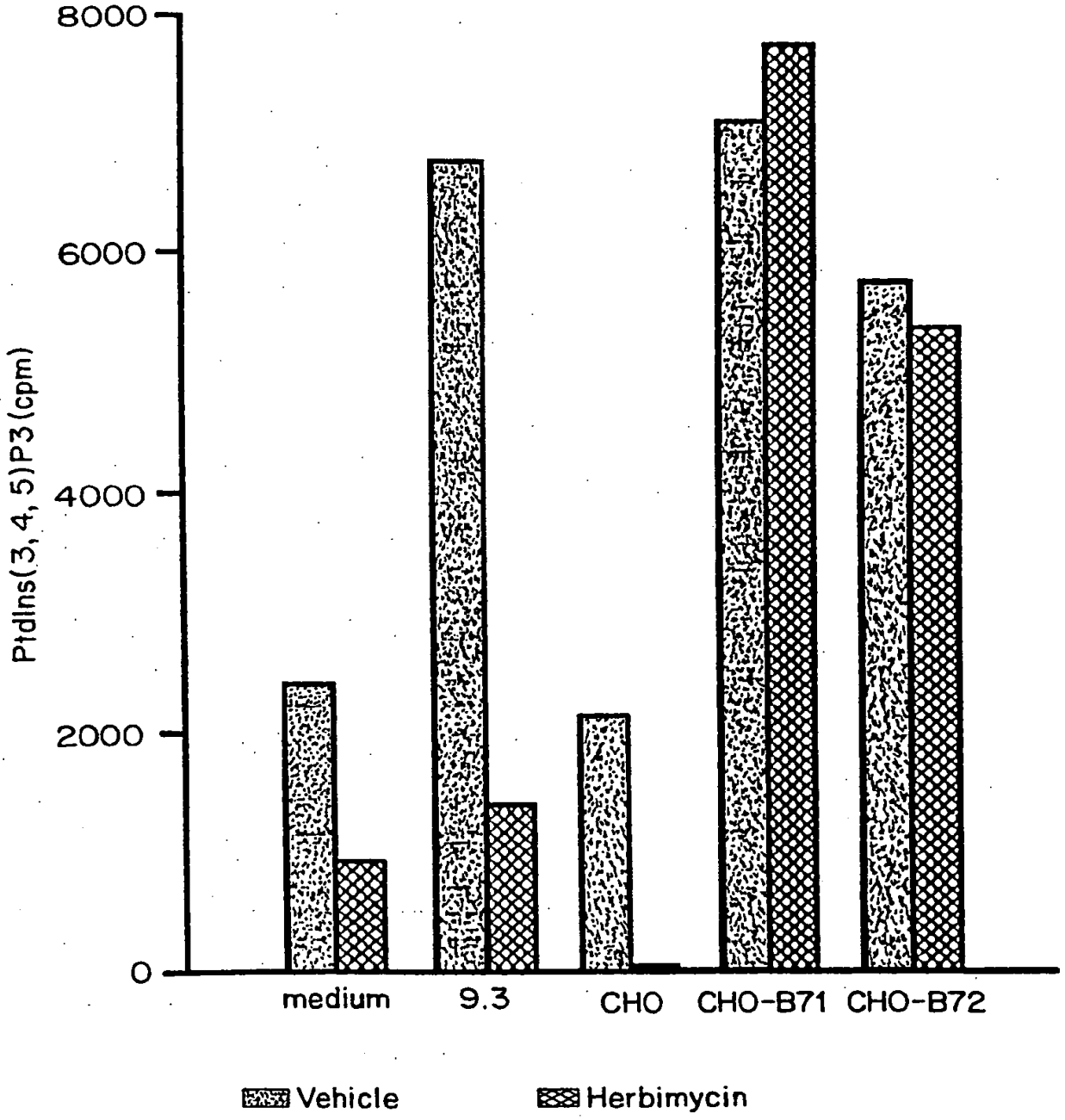
OKT3+CHO-B72
OKT3+CHO-B71+B72
PMA+CHO-B71
PMA+CHO-B72

FIG. 8


8000
6000
4000
2000
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medium
9.3
CHO
CHO-B71
CHO-B72
Vehicle
Herbimycin

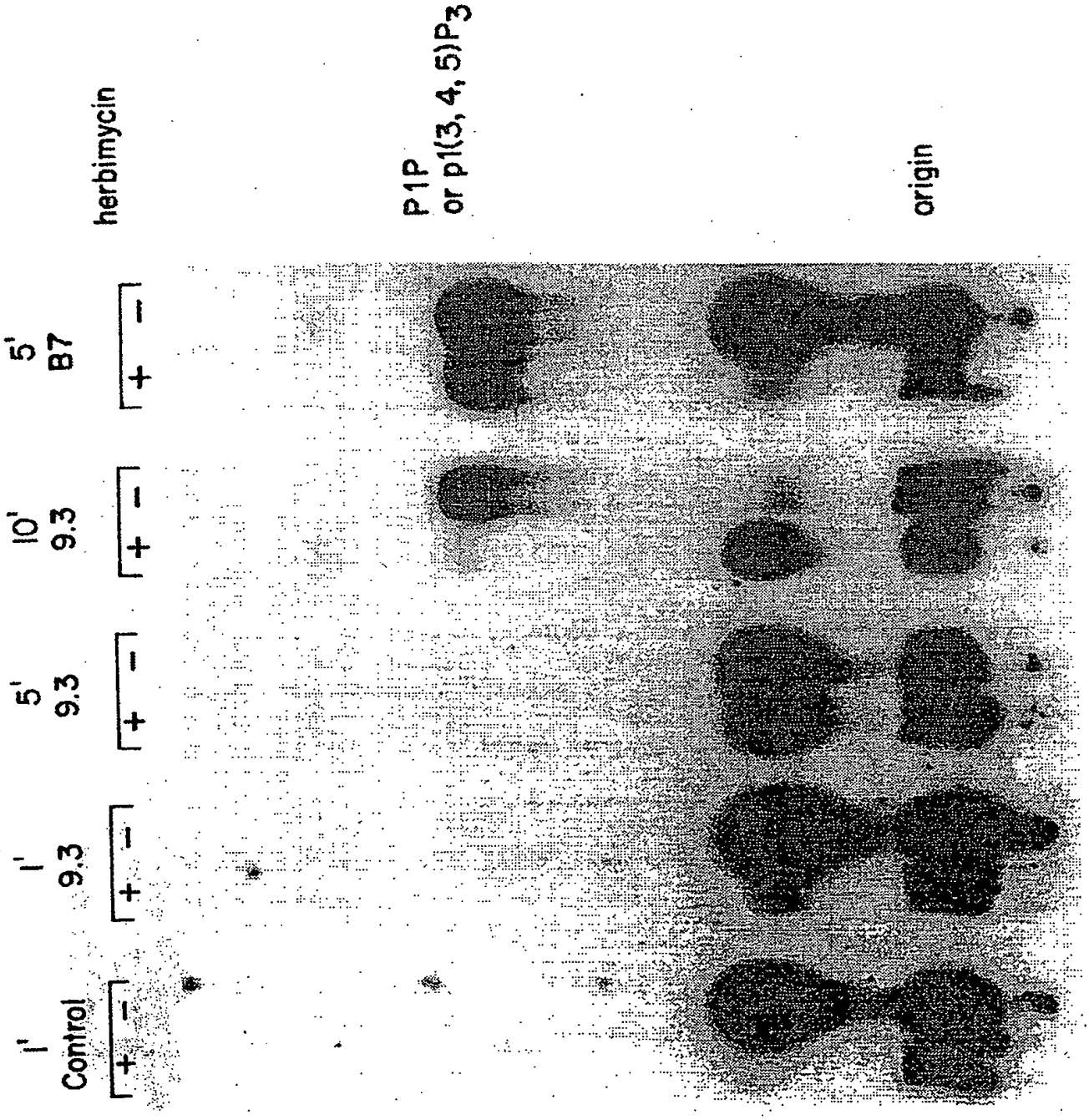


FIG. 9

FIG. 10A

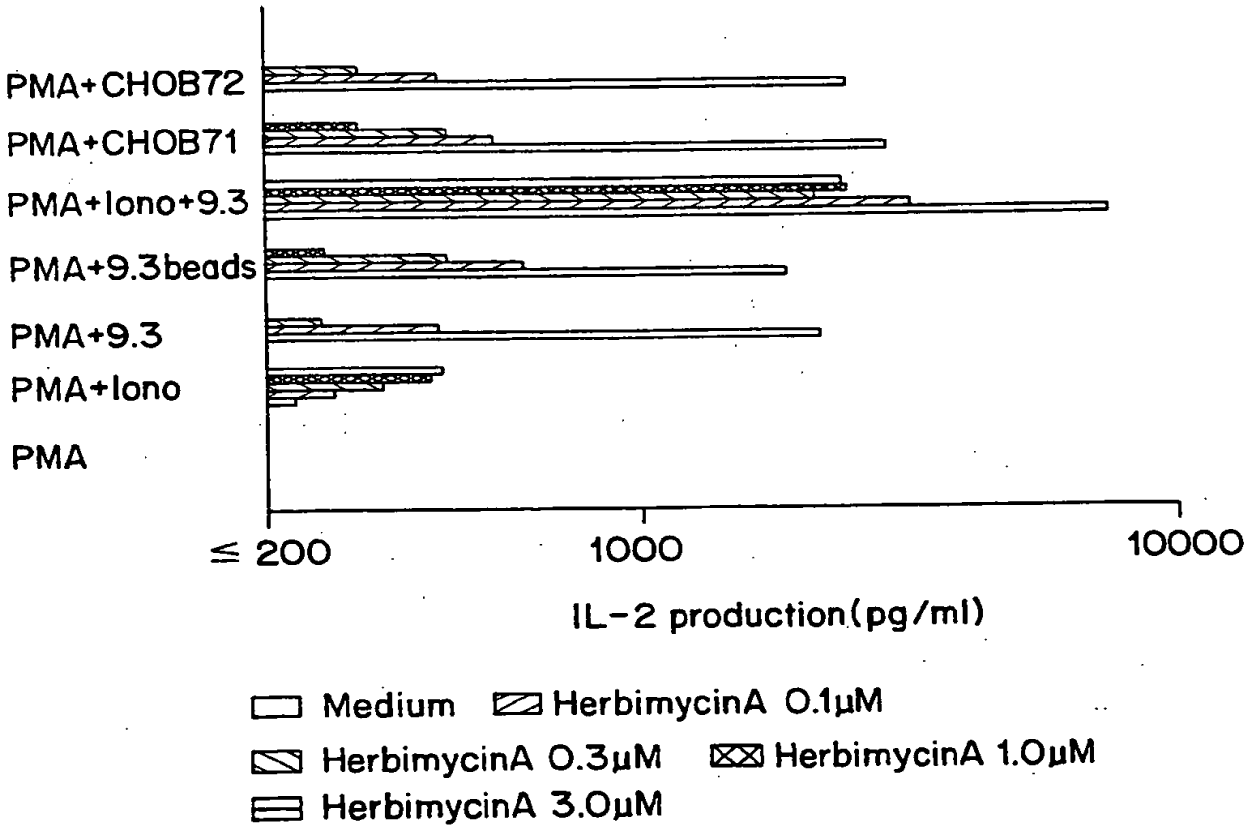


FIG. 10B

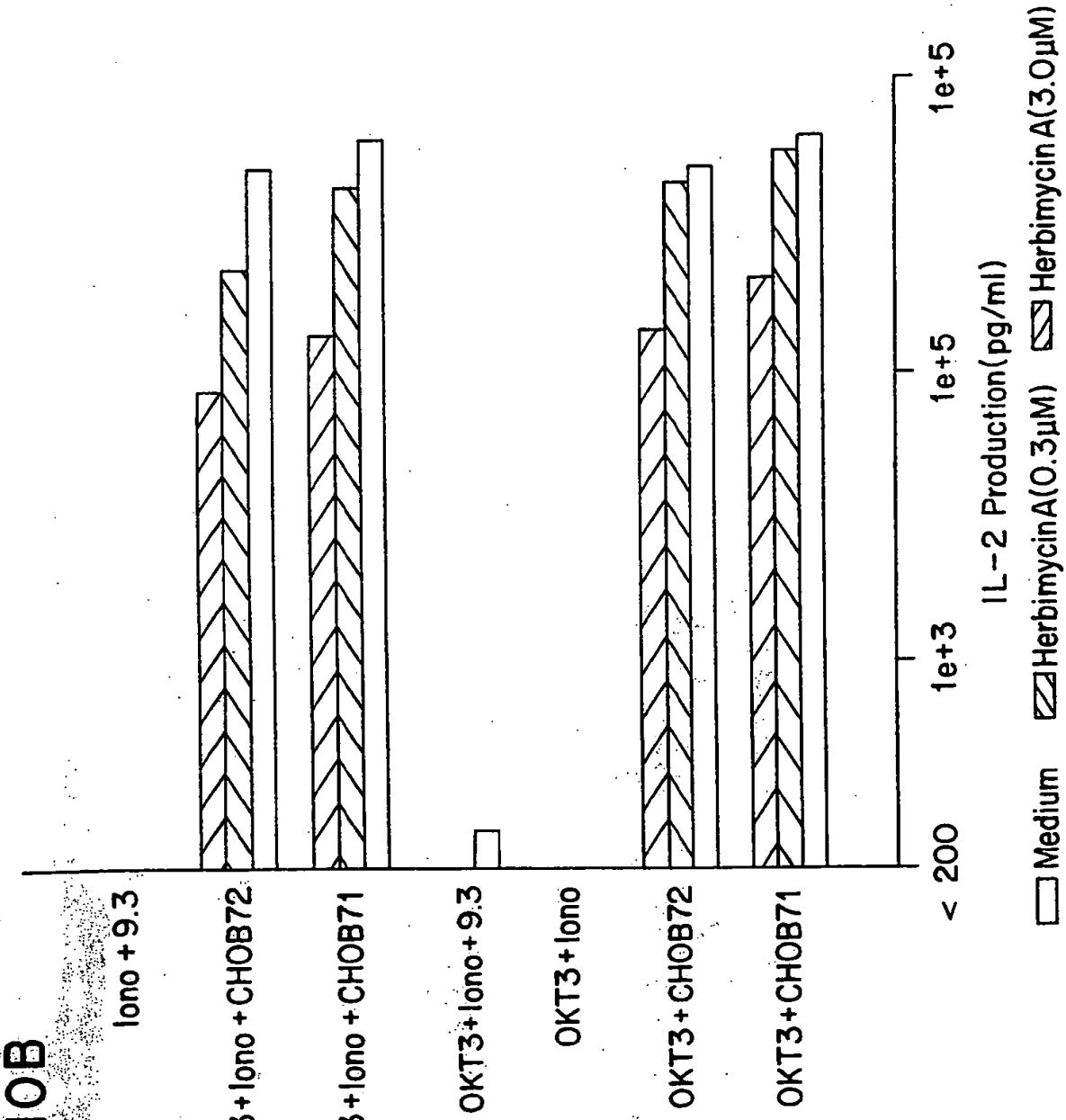

Iono +9.3
OKT3+Iono+CHOB72
OKT3+Iono+CHOB71
OKT3+Iono+9.3
OKT3+Iono
OKT3+CHOB72
OKT3+CHOB71
< 200
1e+3
1e+5
1e+5
IL-2 Production(pg/ml)
Medium
HerbimycinA(0.3μM)
HerbimycinA(3.0μM)

FIG. 11

INTERNATIONAL SEARCH REPORT

Inter mal Application No
PCT/US 95/05213

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/365 A61K31/35 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,95 10628 (DANA-FARBER CANCER INSTITUTE) 20 April 1995 see page 1, paragraph 1 see page 3, line 4 - line 17 see page 4, line 31 see page 21; example 1 see page 24; example 2 see page 23, line 13 - page 24, line 90 see claim 1 --- -/--	1-55

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 August 1995	21.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gerli, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/05213

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	EUR.J.IMMUNOL., vol. 25, no. 2, February 1995 pages 526-32, 'Inhibition of CD28 mediated T cell costimulation by the phosphoinositide 3-kinase inhibitor wortmannin' see abstract see page 526, right column, line 35 - page 527, left column, line 5 see page 531, left column, paragraph 3 ---	1-55
X	CANCER RES., vol. 52, no. 23, 1992 pages 6676-81, 'Quercetin arrests human leukemic T-cells in late G1 phase of the cell cycle' see page 6676, right column, line 7 - line 21 see page 6677, left column, paragraph 2 see page 6679, right column, line 13 - line 19 ---	1-55
X	IMMUNOPHARMACOLOGY, vol. 4, no. 2, 1982 pages 125-38, 'Quercetin inhibition of the induction and function of cytotoxic T lymphocytes' see abstract see page 126, line 5 - line 9 see page 128, paragraph 2 see page 130, paragraph 2 see page 132, line 9 - page 134, line 10 see page 137, line 29 - line 34 ---	1-55
X	POULT.SCI., vol. 71, no. suppl.1, 1992 page 13. see abstract no.2 -----	1-55

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05213

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark:Although claims 1-38, 46-55 are directed to a method of treatment of (diagnostic method practised on)the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 56
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The protein of claim 56 could not be searched because it is not chemically characterised.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/US 95/05213

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9510628	20-04-95	NONE	

FIG. 1

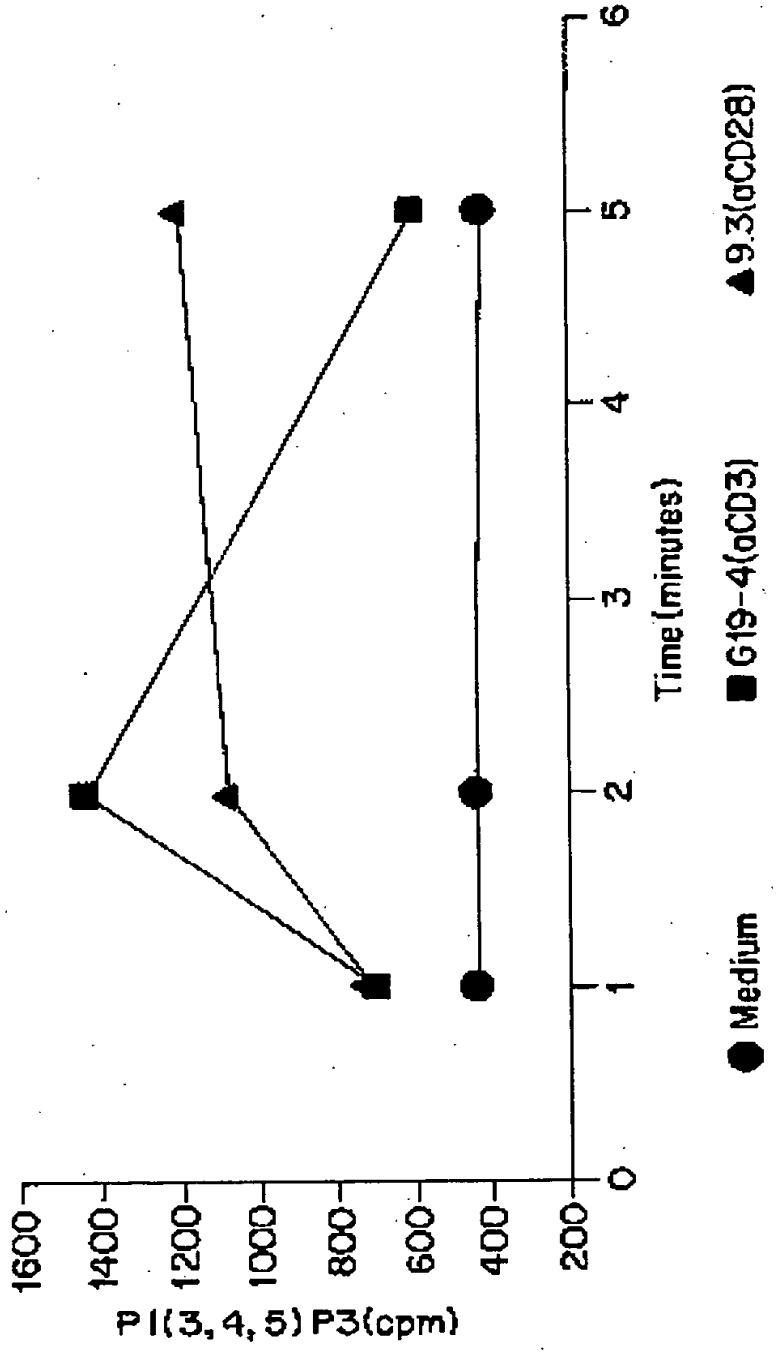

1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
PI(3, 4, 5) P3(cpm)
Time (minutes)
Medium
G19-4(αCD3)
9.3(αCD28)

FIG. 2

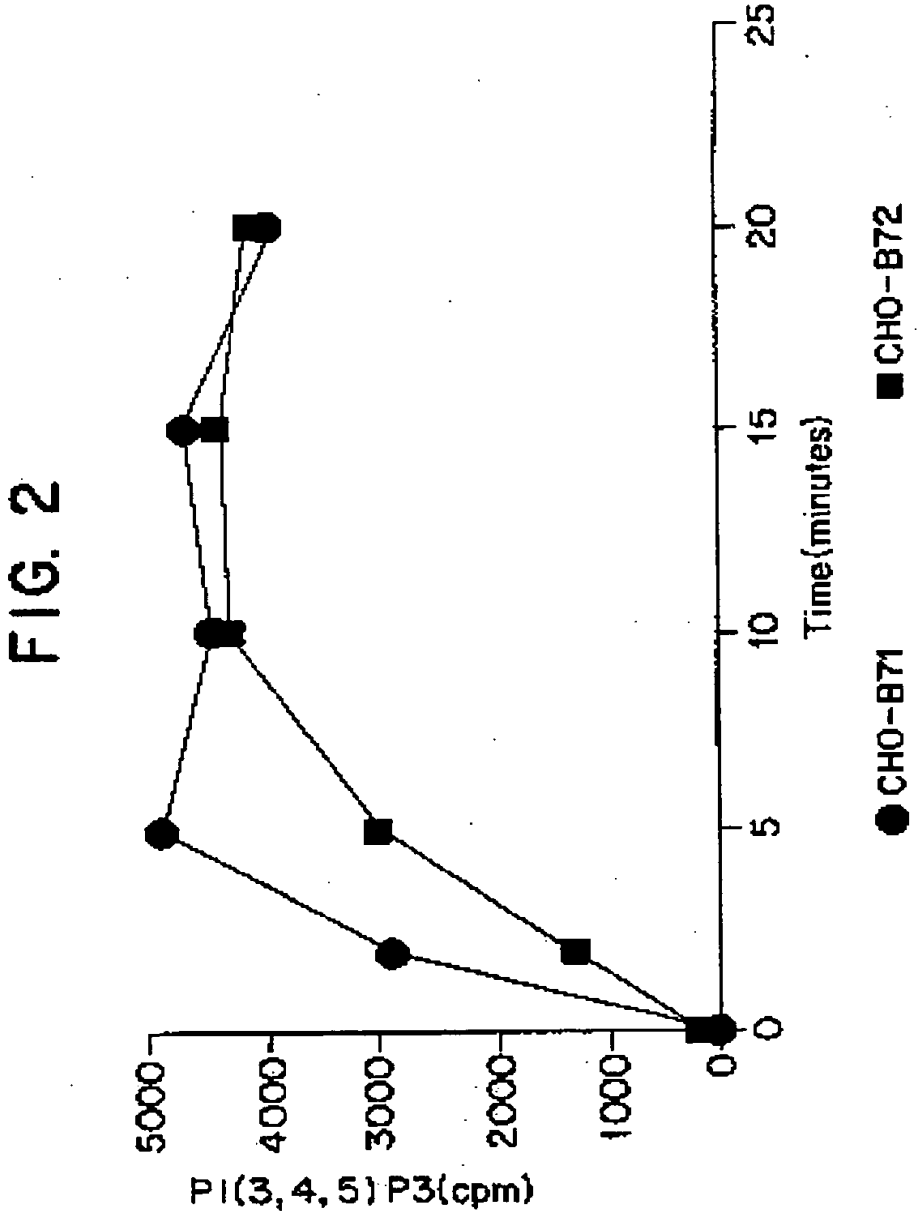

PI(3,4,5)P3(cpm)
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
Time(minutes)
CHO-B71
CHO-B72

FIG. 3

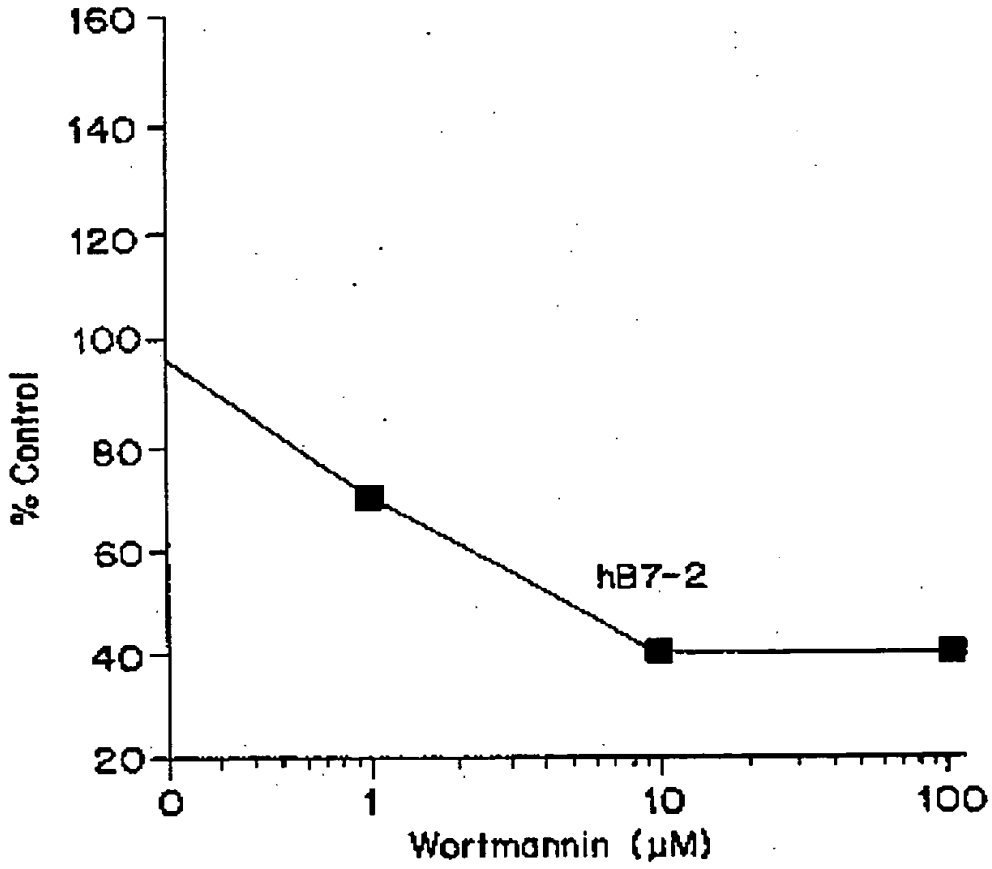
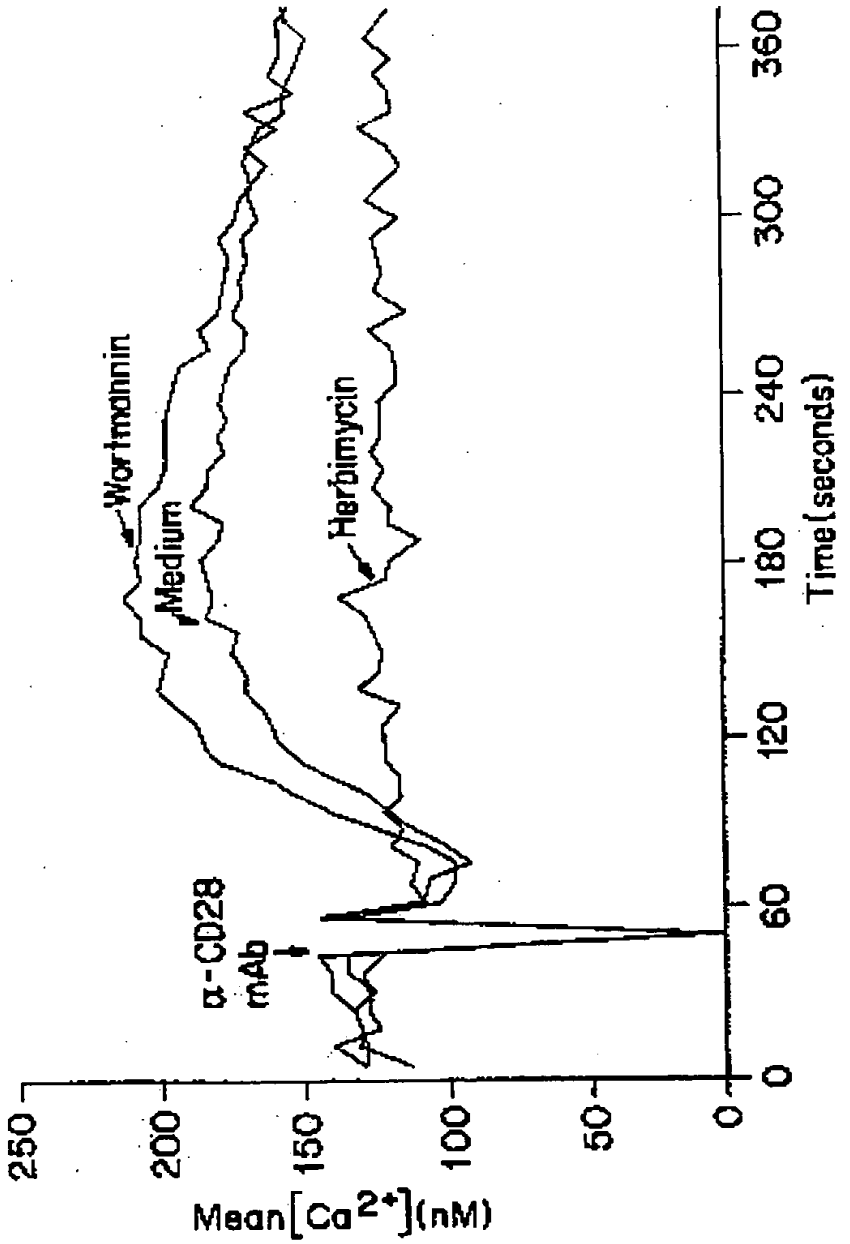


FIG. 4


Wortmannin
Medium
Herbimycin
α-CD28 mAb
Mean[Ca2+](nM)
Time(seconds)
250
200
150
100
50
0
0
60
120
180
240
300
360

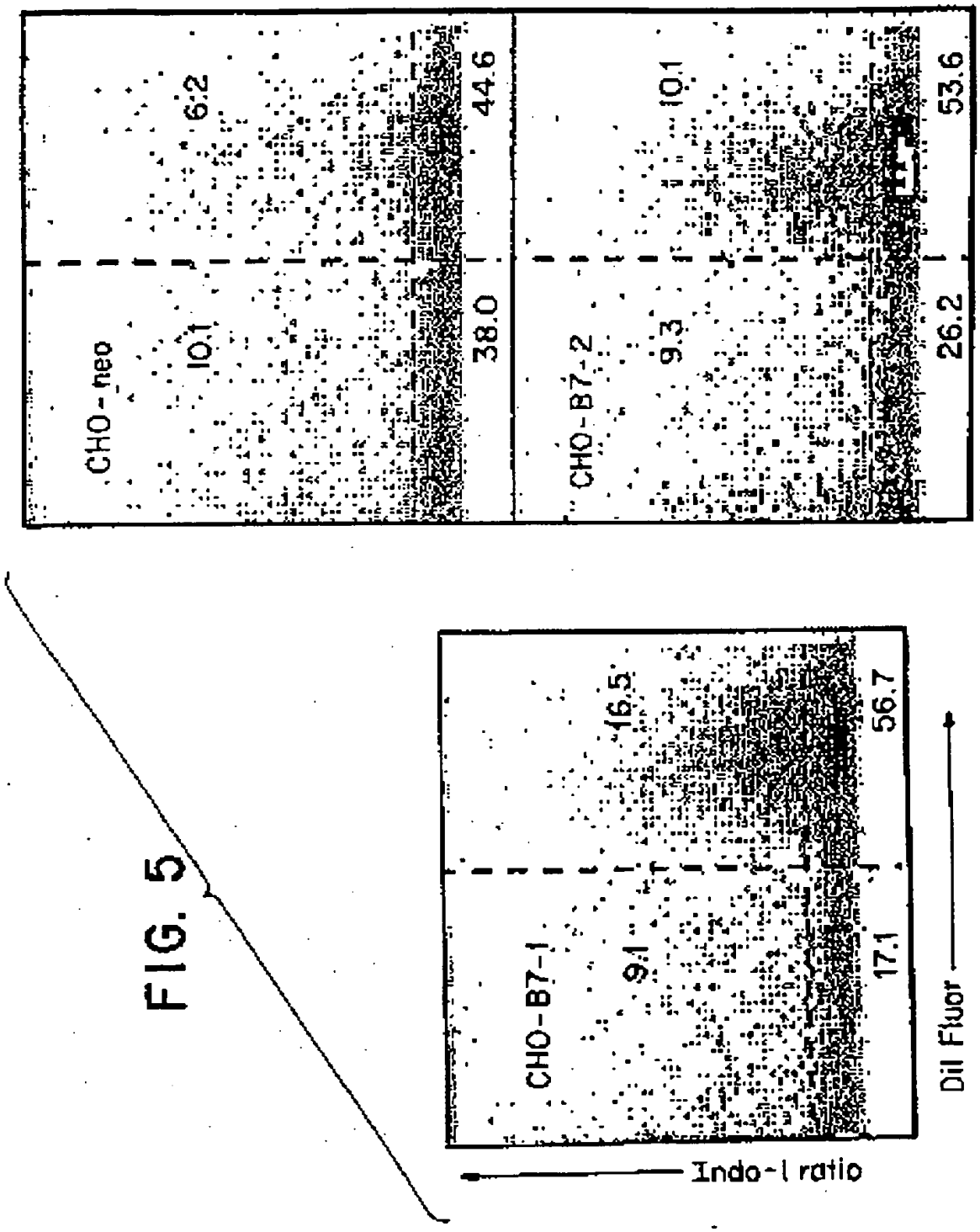


FIG. 5

FIG. 6

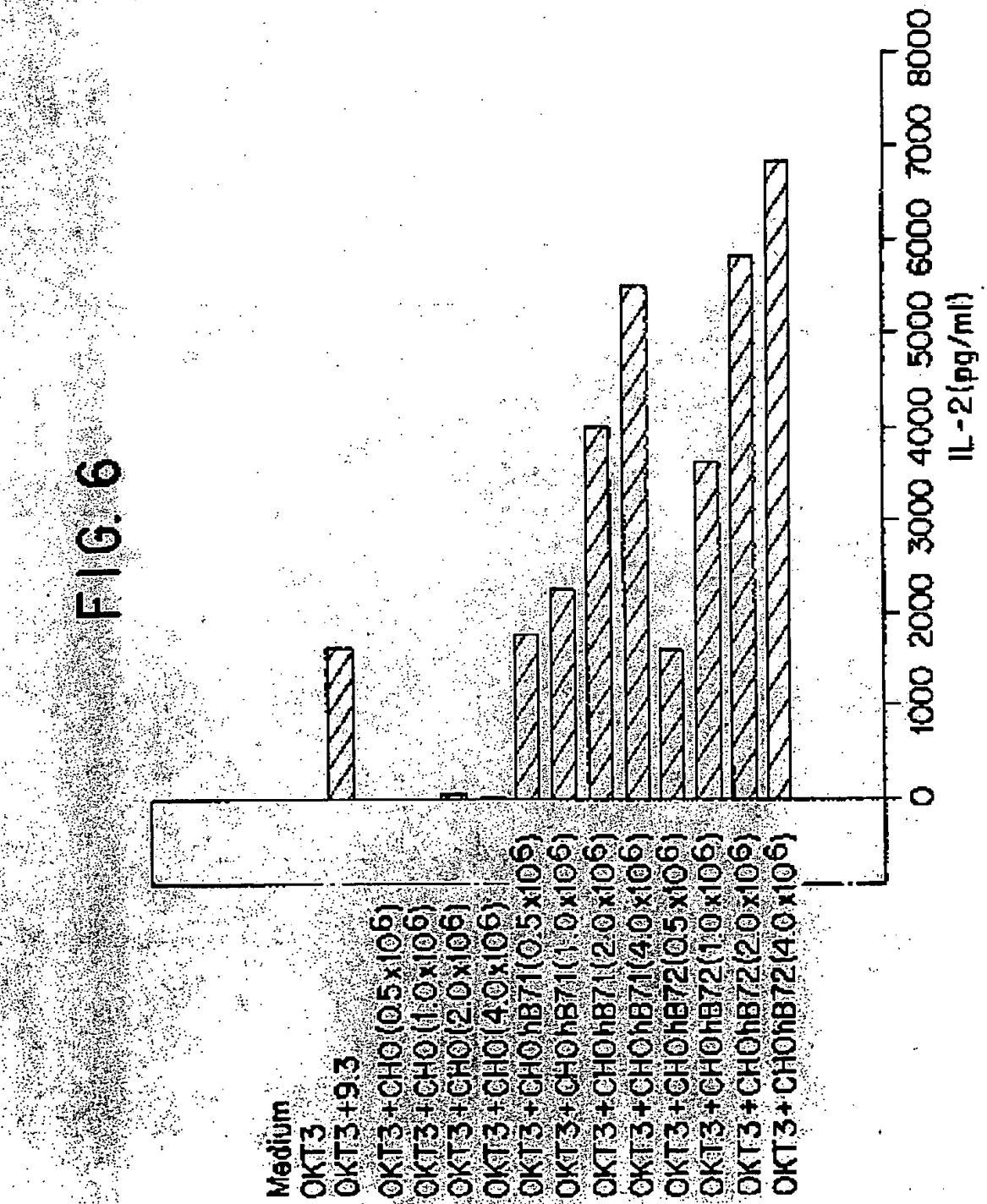

IL-2(pg/ml)
0
1000
2000
3000
4000
5000
6000
7000
8000
Medium
OKT3
OKT3+9.3
OKT3+CHO(0.5x106)
OKT3+CHO(1.0x106)
OKT3+CHO(2.0x106)
OKT3+CHO(4.0x106)
OKT3+CHOhB71(0.5x106)
OKT3+CHOhB71(1.0x106)
OKT3+CHOhB71(2.0x106)
OKT3+CHOhB71(4.0x106)
OKT3+CHOhB72(0.5x106)
OKT3+CHOhB72(1.0x106)
OKT3+CHOhB72(2.0x106)
OKT3+CHOhB72(4.0x106)

FIG. 7A

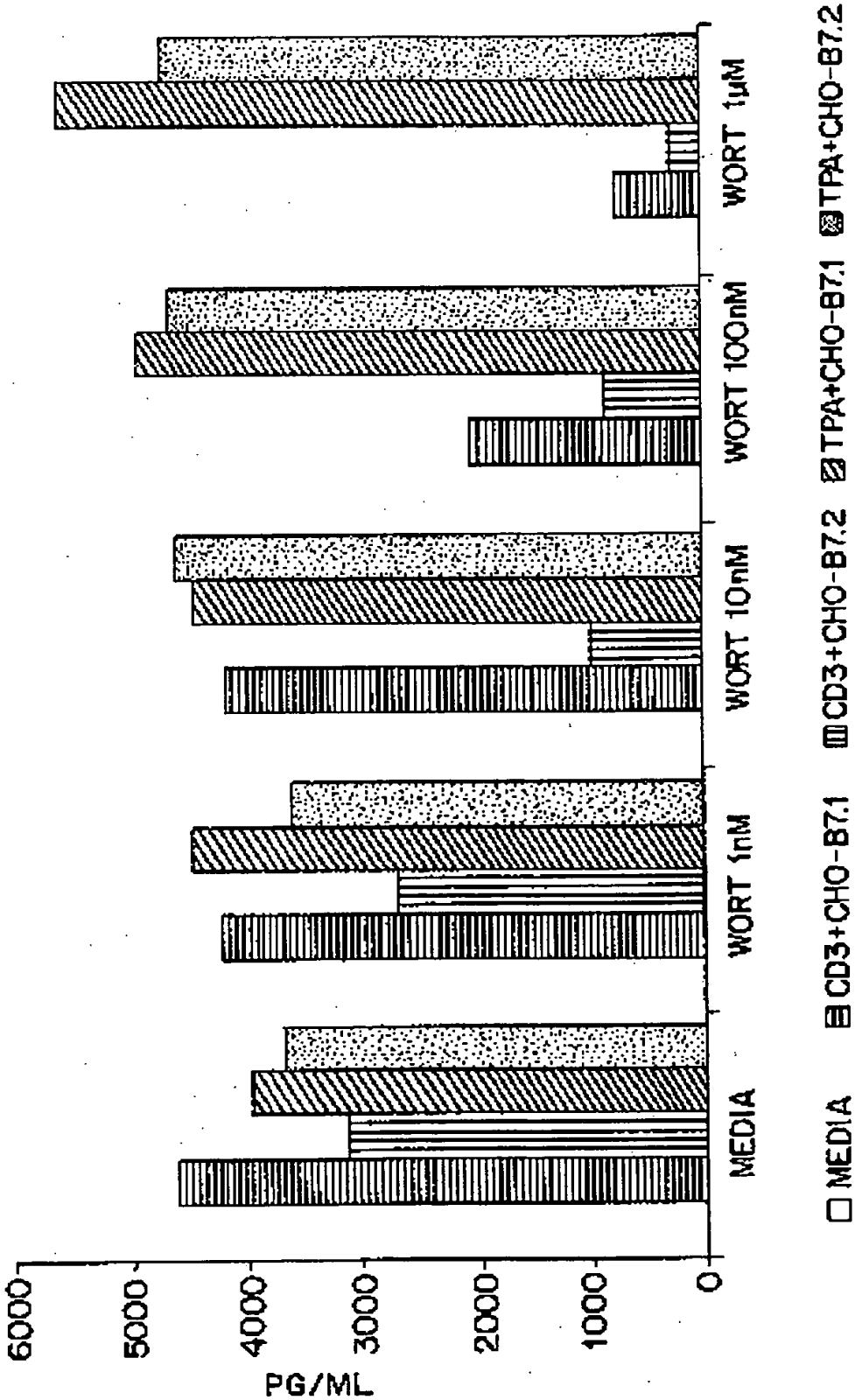

6000
5000
4000
3000
2000
1000
0
PG/ML
MEDIA
WORT 1nM
WORT 10nM
WORT 100nM
WORT 1μM
MEDIA
CD3+CHO-B7.1
CD3+CHO-B7.2
TPA+CHO-B7.1
TPA+CHO-B7.2

FIG. 7B

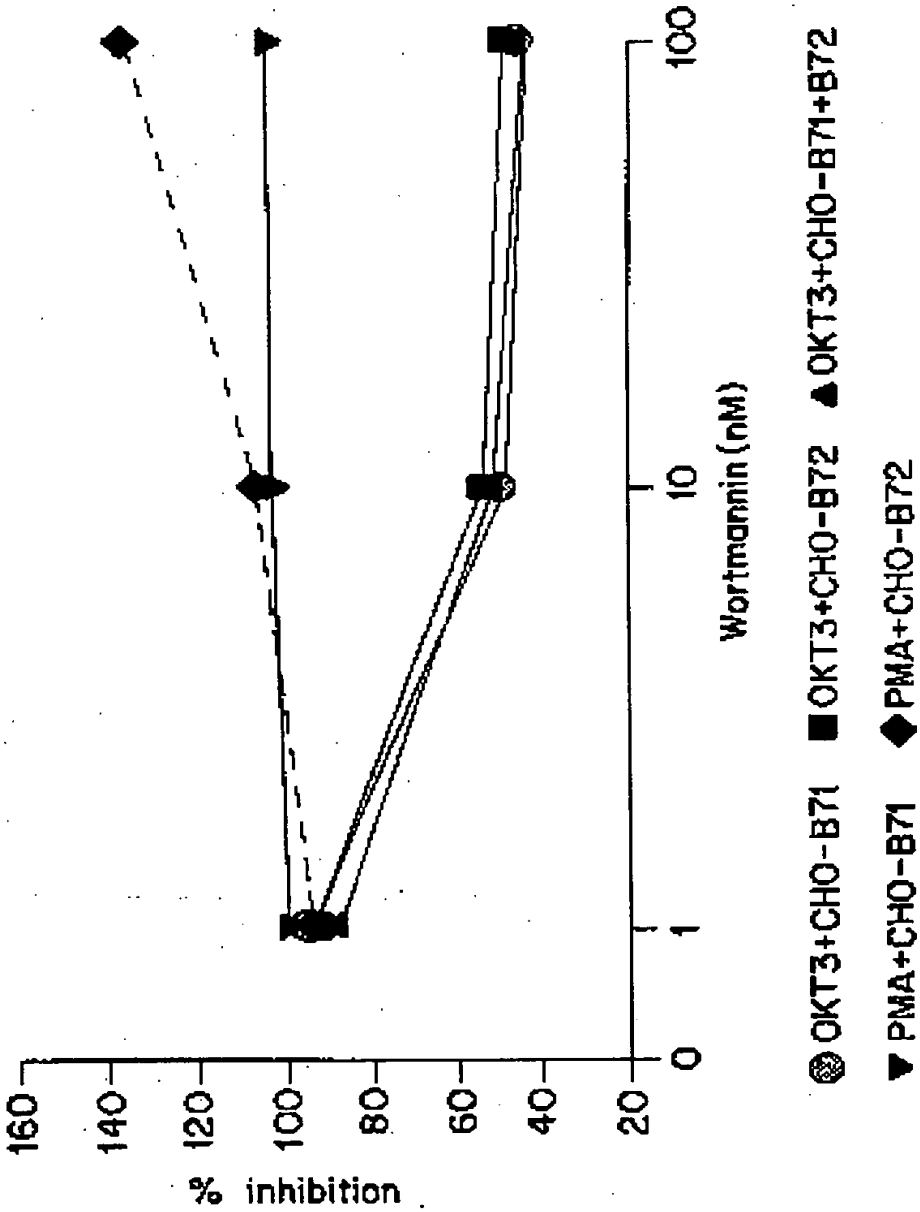
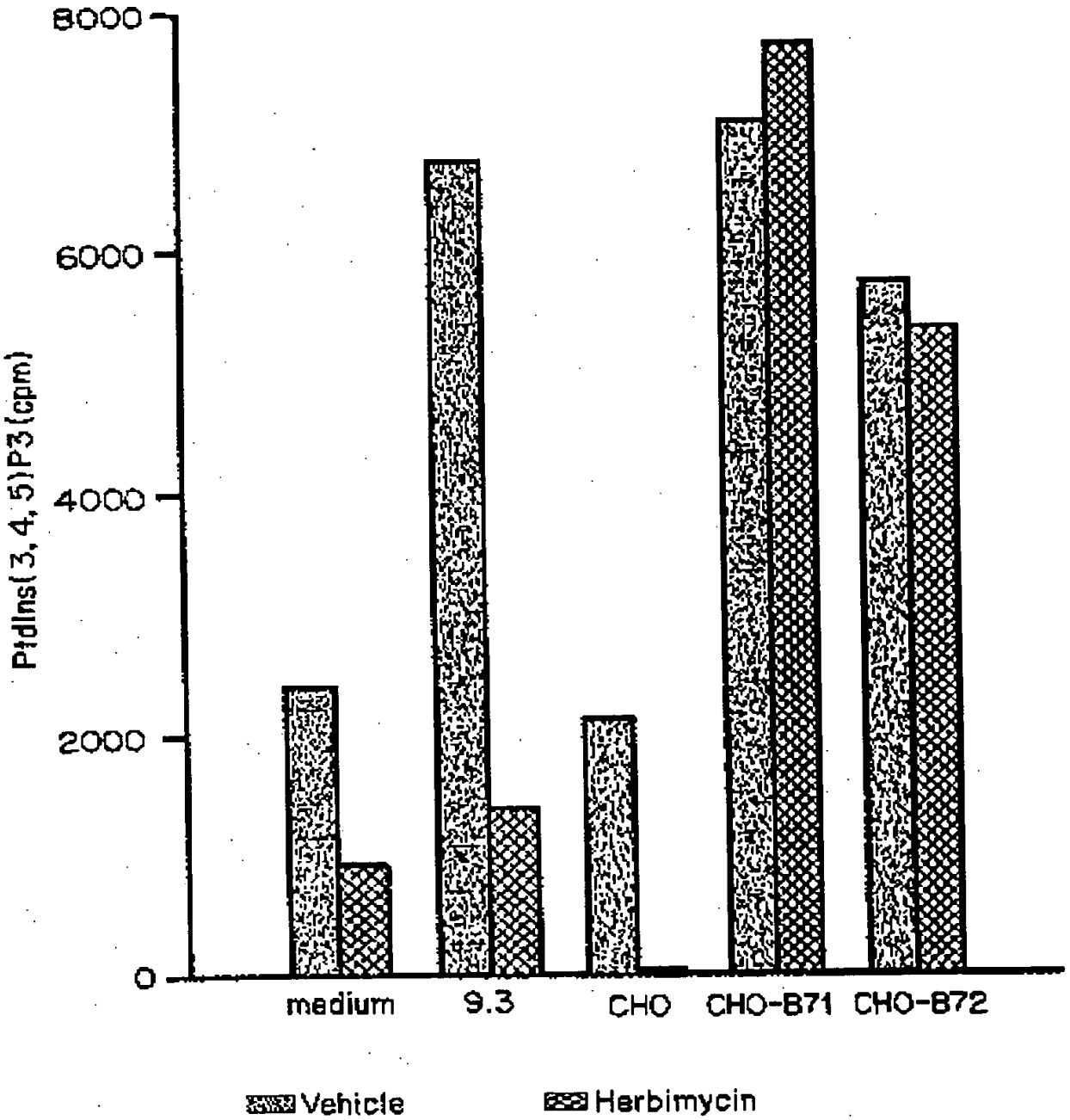

% inhibition
160
140
120
100
80
60
40
20
0
1
10
100
Wortmannin (nM)
OKT3+CHO-B71
OKT3+CHO-B72
OKT3+CHO-B71+B72
PMA+CHO-B71
PMA+CHO-B72

FIG. 8


8000
6000
4000
2000
0
PtdIns(3, 4, 5)P3 (cpm)
medium
9.3
CHO
CHO-B71
CHO-B72
Vehicle
Herbimycin

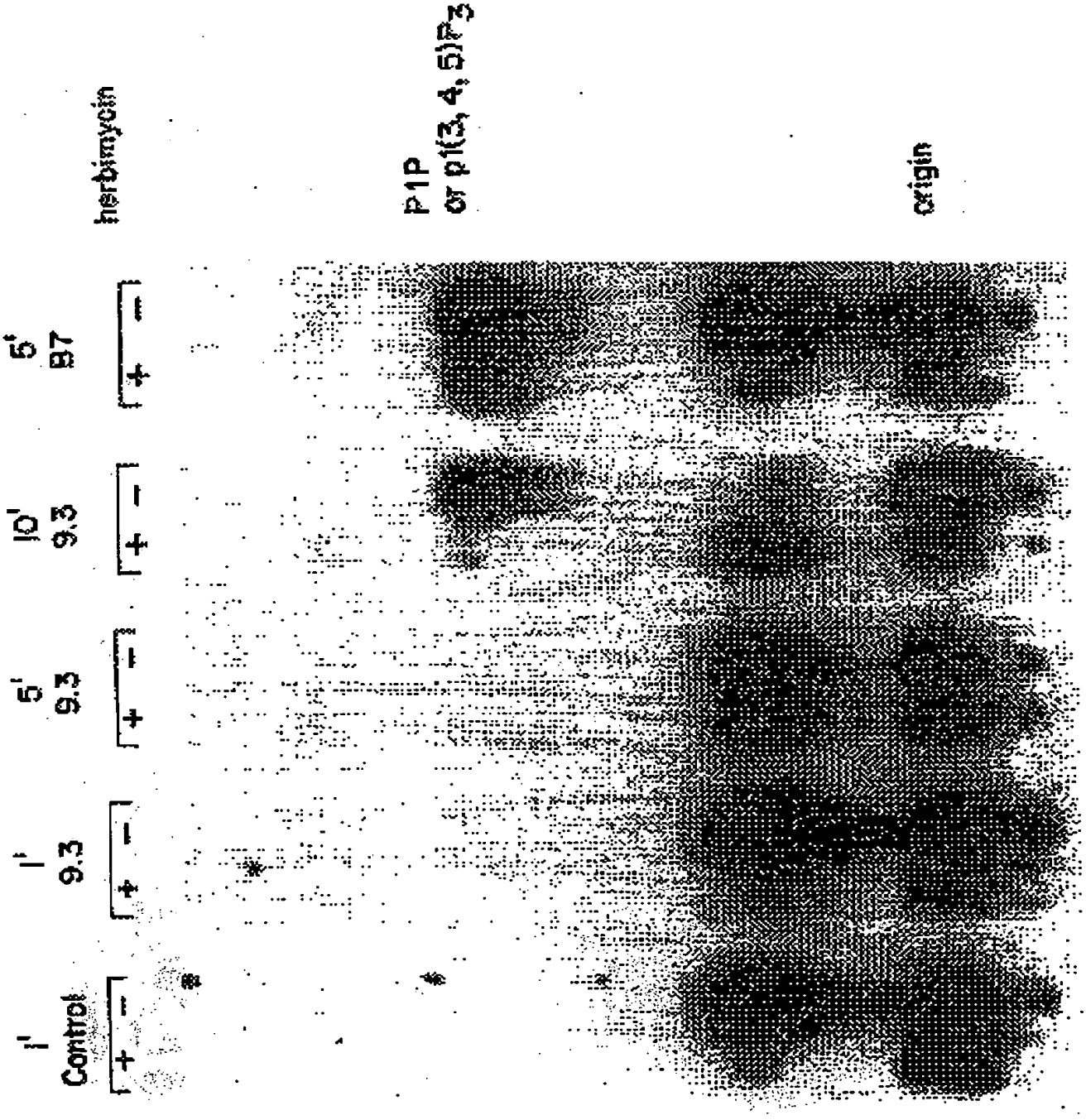


FIG. 9

FIG. 10A

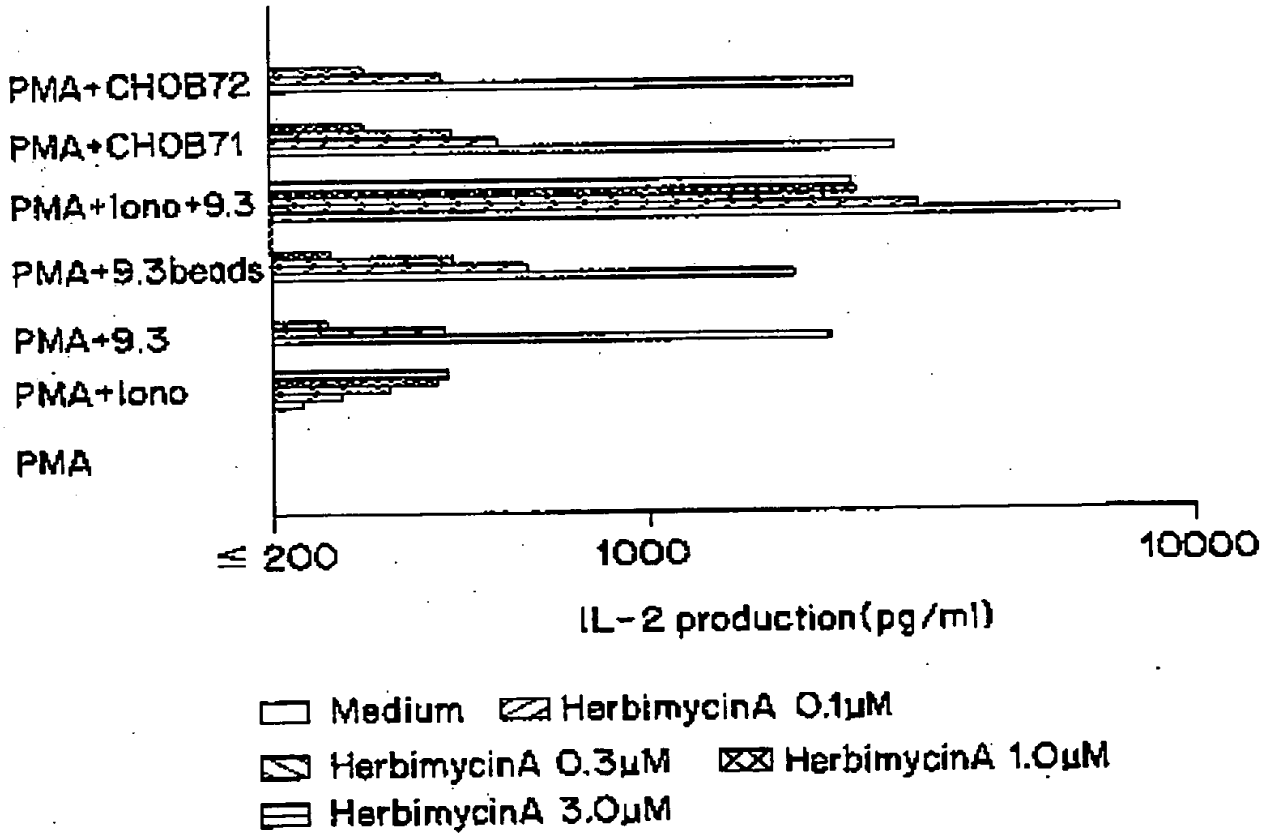
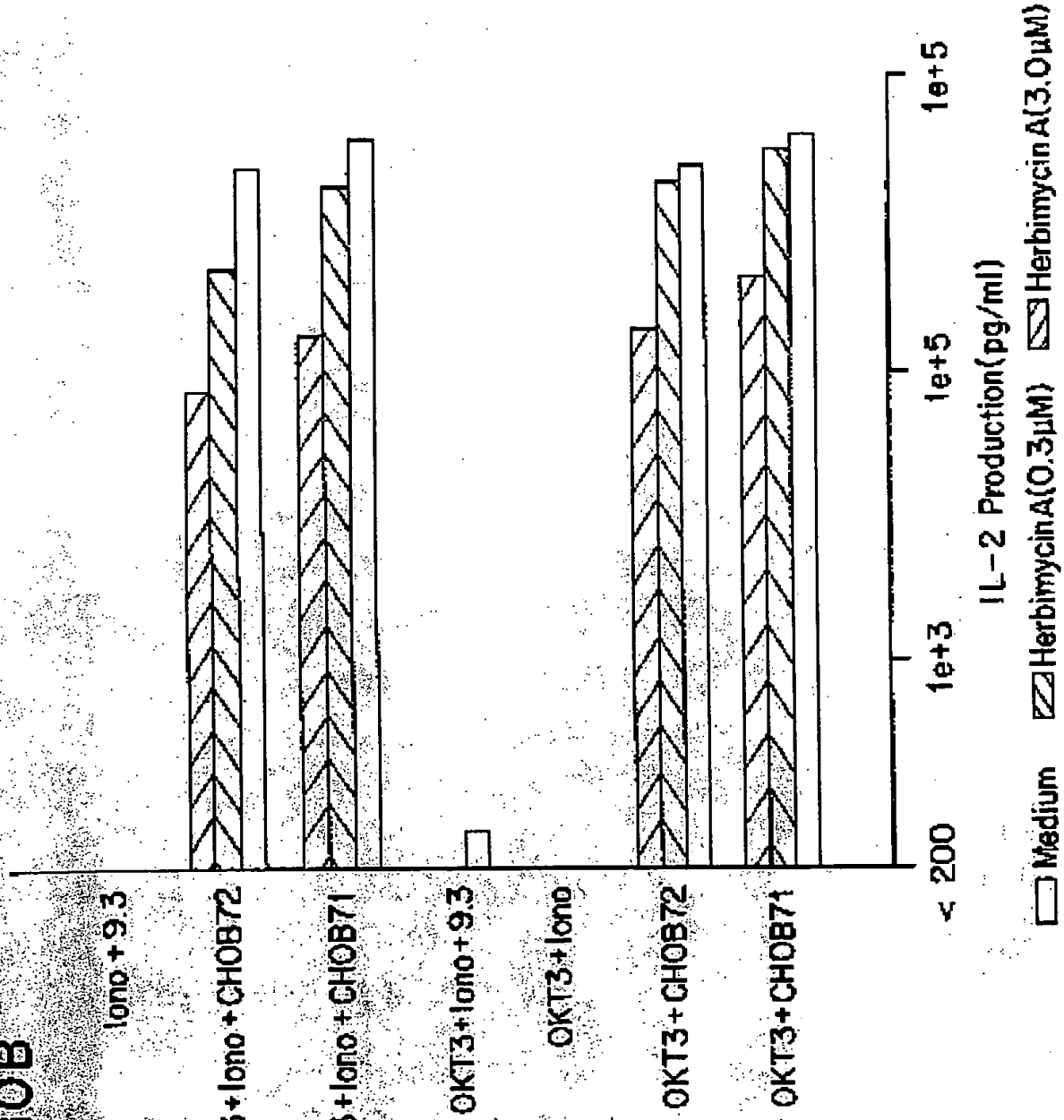

PMA+CHOB72
PMA+CHOB71
PMA+Iono+9.3
PMA+9.3beads
PMA+9.3
PMA+Iono
PMA
≤ 200
1000
10000
IL-2 production(pg/ml)
Medium
HerbimycinA 0.1μM
HerbimycinA 0.3μM
HerbimycinA 1.0μM
HerbimycinA 3.0μM

FIG. 10B


Iono +9.3
OKT3+Iono+CHOB72
OKT3+Iono+CHOB71
OKT3+Iono+9.3
OKT3+Iono
OKT3+CHOB72
OKT3+CHOB71
< 200
1e+3
1e+5
1e+5
IL-2 Production(pg/ml)
Medium
Herbimycin A(0.3μM)
Herbimycin A(3.0μM)

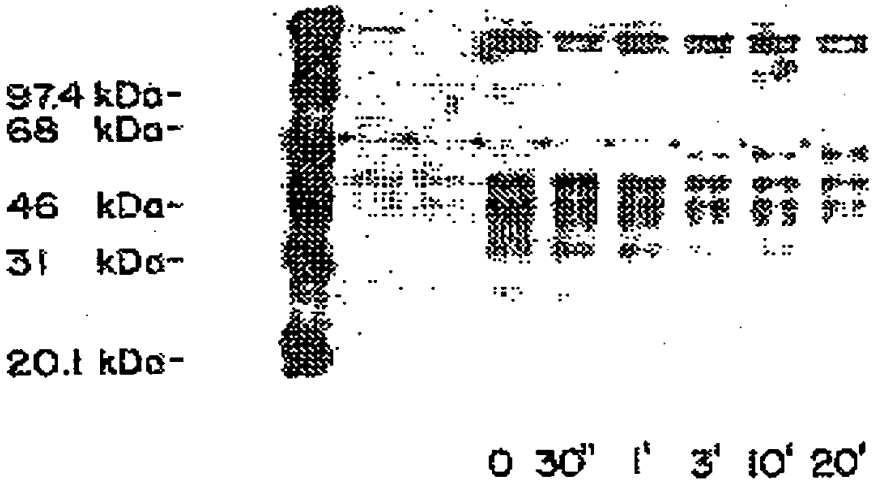


FIG. 11